Prevention of retrogradation of starch

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(54) Title: PREVENTION OF RETROGRADATION OF STARCH

(57) Abstract: The invention provides an isolated or recombinant nucleic acid derived from a nucleic acid encoding a polypeptide essentially having alpha-glucanotransferase activity but having essentially no hydrolysing activity, said isolated or recombinant nucleic acid encoding a polypeptide with hydrolytic activity.

Title: Prevention of retrogradation of starch.

Starch containing crops form an important constituent of the human diet and a large proportion of the food consumed by the world's population originates from them. Besides the use of the starch-containing plant parts directly as a food source, starch is harvested and used as such or chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, or cyclodextrins. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat. In the European Union, 3.6 million tons of maize starch, 2 million tons of wheat starch, and 1.8 million tons of potato starch were produced in 1998 (DeBaere, 1999).

Starch is found as granules containing polymers of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. This glycosidic bond is stable at high pH but hydrolyses at low pH. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end. Two types of glucose polymers are present in starch granules: (i) amylose and (ii) amylopectin. Amylose is a linear polymer consisting of up to 6,000 glucose units with alpha,1-4 glycosidic bonds. The number of glucose residues, also indicated with the term DP (degree of polymerization), varies with the origin. Amylose from e.g. potato or tapioca starch has a DP of 1,000 – 6,000 while amylose from maize or wheat amylose has a DP varying between 200 and 1,200. The avarage amylose content in starches can vary between almost 0 to 75%, but a typical value is 20-25%. Amylopectin consists of short alpha,1-4 linked linear chains of 10-60 glucose units and alpha,1-6 linked side chains with 15-45 glucose units. The average number of branching points in amylopectin is 5%, but varies with the botanical origin. The complete amylopectin molecule contains on avarge about 2,000,000 glucose units, thereby being one of the largest molecules in nature. The most commonly accepted model of the structure of amylopectin is the cluster model, in which the side chains are ordered in clusters on the longer backbone chains (see Buléon et al., 1998; Myers et al., 2000).

While amylopectin is soluble in water, amylose and the starch granule itself are insoluble in cold water. This makes it relatively easy to extract starch granules from

their plant source. When a water-starch slurry is heated, the granules first swell untill a point is reached at which the swelling is irreversible. This swelling processes is termed gelatinization. During this process, amylose leaches out of the granule and causes an increase in the viscocity of the slurry. Further increase in temperature then leads to maximum swelling of the granules and increased viscosity. Finally, the granules break apart resulting in a complete viscous colloidal dispersion. Subsequent cooling results in association of the amylose chains, resulting in the formation of insoluble aggregates. In diluted starch suspensions these aggregates precipitate, cooling of a concentrated colloidal starch dispersion results in the formation of an elastic gel. This retrogradation is primarily caused by the amylose; amylopectin, due to its highly branched organization, is less prone to retrogradation.

A large variety of bacteria employ extracellular or intracellular enzymes able to convert starch or glycogen, that thus can serve as energy and carbon sources (Fig. 2).

There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases.

Endoamylases are able to cleave alpha,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. alpha-Amylase (EC 3.2.1.1) is a well known endoamylase. It is found in a wide variety of microorganisms, belonging to the Archaea as well as the Bacteria (Pandey et al., 2000). The end products of alpha-amylase action are oligosaccharides with varying length with an alpha-configuration and alpha-limit dextrins, which constitute branched oligosaccharides.

Enzymes belonging to the second group, the exoamylases, either exclusively cleave alpha,1-4 glycosidic bonds such as b-amylase (EC 3.2.1.2) or cleave both alpha,1-4 and alpha,1-6 glycosidic bonds like amyloglucosidase or glucoamylase (EC 3.2.1.3) and alpha-glucosidase (EC 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and alpha-glucosidase), or maltose and b-limit dextrin (b-amylase).

The third group of starch-converting enzymes are the debranching enzymes that exclusively hydrolyse alpha,1-6 glycosidic bonds: isoamylase (EC 3.2.1.68) and pullanase type I (EC 3.2.1.41). Pullulanases hydrolyse the alpha,1-6 glycosidic bond in pullulan and amylopectin, while isoamylase can only hydrolyse the alpha,1-6 bond in amylopectin. These enzymes exclusively degrade amylopectin, thus leaving long linear polysaccharides.

There are also a number of pullulanase type enzymes that hydrolyse both alpha,1-4 and alpha,1-6 glycosidic bonds. These belong to the group II pullulanase and are also referred to as alpha-amylase-pullulanase or amylopullulanase. The main degradation products are maltose and maltotriose.

The fourth group of starch-converting enzymes are transferases that cleave an alpha,1-4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) form a new alpha,1-4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new alpha,1,6 glycosidic bond.

Cyclodextrin glycosyltransferases have a very low intrinsic hydrolytic activity and make cyclic oligosaccharides with 6,7,or 8 glucose residues and highly branched high molecular weight dextrins, the cyclodextrin glycosyl-transferase limit dextrins. Cyclodextrins are produced via an intramolecular transglycosylation reaction in which the enzyme cleaves an alpha,1-4 glycosidic bond and concomitantly links the reducing to the non-reducing end (Takaha and Smith, 1999; van der Veen et al., 2000a).

Amylomaltases are very similar to cyclodextrin glycosyltransferases with respect to the type of enzymatic reaction. The major difference is that amylomaltase performs a transglycosylation reaction resulting in a linear product while cyclodextrin glycosyl-transferase gives a cyclic product. Another difference is that they essentially do not hydrolyse starch. Amylomaltases have been found in different microorganisms in which it is involved in the utilization of maltose or the degradation of glycogen (Takaha and Smith, 1999).

Glucan branching enzymes are invloved in the synthesis of glycogen in many microorganisms. They are responsible for the formation of alpha,1-6 glycosidic bonds in the side chains of glycogen and in general do not hydrolyse either. Although glycogen has been found in a large number of microorganisms (Preiss, 1984), only a limited number of microbial glucan branching enzymes have been characterized (Kiel et al., 1991; Kiel et al., 1992; Takata et al., 1994; Binderup and Preiss, 1998).

Most of the enzymes mentioned above belong to one family based on amino acid sequence homology: the alpha-amylase family or family 13 glycosyl hydrolases according to the classification of Henrissat (1991). This group comprises those enzymes that have the following features: (i) they possess a (bete/alpha)$_8$ or TIM

barrel (Fig. 3) structure containing the catalytic residues; (ii) they have four highly conserved regions in their primary sequence (Table 1) which contain the amino acids that form the catalytic site, as well as some amino acids that are essential for the stability of the conserved TIM barrel topology (Kuriki and Imanaka, 1999); (iii) they act on alpha-glycosidic bonds and hydrolyse or transglycosylate this bond with retention of the alpha-anomeric configuration through a double displacement mechanism.

The enzymes that match the above mentioned criteria and belong to the alpha-amylase family are listed in Table 2.

During the last three decades, alpha-amylases have been exploited by the starch-processing industry as a replacement of acid hydrolysis in the production of starch hydrolysates. This enzyme is also used for removal of starch in beer, fruit juices, or from clothes and porcelain. Another starch-hydrolysing enzyme that is used on a large scale is thermostable pullulanase for the debranching of amylopectin. A recent application is directed at the use of maltogenic amylases as an anti-staling agents to prevent the retrogradation of starch in bakery products.

The baking industry is a large consumer of starch and starch modifying enzymes. Bread baking starts with dough preparation by mixing flour, water, yeast and salt and possibly additives. Flour consists mainly of gluten, starch, non-starch polysaccharides and lipids. Immediately after dough preparation, the yeast starts to ferment the available sugars into alcohols and carbon dioxide, which causes rising of the dough. Amylases can be added to the dough to degrade the damaged starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of malt or fungal α-amylase to the dough results in increased loaf volume and improved texture of the baked product

After rising, the dough is baked. When the bread is removed from the oven, a series of changes start which eventually leads to deterioration of quality. These changes include increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor. All undesirable changes that do occur upon storage together are called staling. Retrogradation of the starch fraction in bread is considered to be very important in staling (Kulp and Ponte, 1981). Especially the extent of amylopectin retrogradation strongly correlates with the firming rate of bread (Champenois et al., 1999). Staling is of considerable economic

importance for the baking industry since it limits the shelf life of baked products. In the USA, for instance, bread worth more than 1 billion US$ is discarded annually.

To delay staling, to improve texture, volume and flavor of bakery products, several additives may be used in bread baking. These include chemicals, small sugars, enzymes or combinations of these. Well known additives are: milk powder, gluten, emulsifiers (mono- or diglycerides, sugar esters, lecithin etc.), granulated fat, oxidant (ascorbic acid or potassium bromate), cysteine, sugars or salts (Spendler and Jørgensen, 1997). Rapid advances in biotechnology have made "new" enzymes available for the industry. Since enzymes are produced from natural ingredients, they will find greater acceptance by the consumers as they demand for products without chemicals. Several enzymes have been suggested to act as dough and/or bread improvers, by modifying one of the major dough components. Examples are glucose oxidase, hemicellulase, lipase, protease and xylanase. These enzymes, however, do not act on the starch fraction itself. Enzymes active on starch have been suggested to act as anti-staling agents. Examples are: α-amylases (De Stefanis and Turner, 1981; Cole, 1982), branching (Okada et al., 1984) and debranching (Carroll et al., 1987) enzymes, maltogenic amylases (Olesen, 1991), β-amylases (Würsch and Gumy, 1994), and amyloglucosidases (Vidal and Gerrity, 1979). Present nti-staling agents, however, often act to fast.

Originally, α-amylases were added during dough preparation to generate fermentable compounds. Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods (De Stefanis and Turner, 1981; Cole, 1982; Sahlström and Bråthen, 1997). Despite a possible anti-staling effect, the use of α-amylases as anti-staling agent is not widespread because even a slight overdose of α-amylase results in a sticky bread. Positive effects of delayed staling, on the contrary, are measured only after 3 to 4 days (Olesen,1991). The increased gummyness of α-amylase treated bread is associated with the production of branched maltodextrins of DP20-100 (De Stefanis and Turner, 1981). Debranching enzymes are claimed to strongly decrease the problems associated with the use of α-amylases as anti-staling agents in baking. In this method a thermostable pullulanase, and an α-amylase are used together. The pullulanase rapidly hydrolyzes the branched maltodextrins of DP20-100 produced by the α-amylase, while they have little effect upon the amylopectin itself (Carroll et al.,

1987). Pullulanase thus specifically removes the compound responsible for the gummyness associated with α-amylase treated bakery products.

Branching enzyme is claimed to increase shelf life and loaf volume of baked goods (Okada et al., 1984; Spendler and Jorgensen, 1997). These effects are achieved by modifying the starch material in the dough during baking. Improved quality of baked products is also obtained when the branching enzyme is used in combination with other enzymes, such as α-amylase, maltogenic amylase, cyclodextrin glycosyltransferase, β-amylase, cellulase, oxidase and/or lipase (Spendler and Jorgensen, 1997).

The use of cyclodextrin glycosyltransferase as dough additive is claimed to increase loaf volume of the backed product (Van Eijk and Mutsaers, 1995). The effect is suggested to result from the gradual formation of cyclodextrins in the dough after mixing.

Exo-amylases, such as β-amylase and amyloglucosidase, shorten the external side chains of amylopectin by cleaving of maltose or glucose molecules, respectively. Both enzymes are suggested to delay bread staling by reducing the tendency of the amylopectin compound in bakery products to retrograde (Würsch and Gumy, 1994). Anti-staling effects of amylo-glucosidase in baking are claimed in a few patents (Van Eijk, 1991; Vidal and Gerrity, 1979). The synergetic use of an α- and a β-amylase is also claimed to increase the shelf life of baked goods (Van Eijk, 1991).

Since α-amylases cause stickiness of backed goods, especially when overdosed, it was suggested that these problems could be solved using an exo-amylase, since they do not produce the branched maltooligosaccharides of DP20-100. Such enzymes, called maltogenic amylases, produce linear oligosaccharides of 2 to 6 glucose residues. Maltogenic amylases producing maltose (Olesen, 1991), maltotriose (Tanaka et al., 1997) and maltotetraose (Shigeji et al., 1999a; Shigeji et al., 1999b) are claimed to increase the shelf life of bakery products by delaying retrogradation of the starch compound. Currently, a thermostable maltogenic amylase of *Bacillus stearothermophilus* (Diderichsen and Christansen, 1988) is used commercially in the bakery industry. Although this enzyme has some endo-activity (Christophersen et al., 1998), it does act as an exo-acting enzyme during baking, modifying starch at a temperature when most of the starch starts to gelatinize (Olesen, 1991).

Cherry et al. (1999) described in detail the 3D-structure of the maltogenic alpha-amylase and used this to suggest specific amino acid modifications to obtain variants of the enzyme with improved product specificity, altered pH optimum, improved thermostability, increased specific activity, altered cleavage pattern and thus have an increased ability to reduce retrogradation of starch or staling of bread.

Cyclodextrins are cyclic alpha,1-4 linked oligosaccharides mainly consisting of 6, 7,or 8 glucose residues. The glucose residues in the rings are arranged in such a manner that the inside is hydrophobic thus resulting in an apolar cavity while the outside is hydrophilic. This enables cyclodextrins to form inclusion complexes with a variety of hydrophobic guest molecules. Specific cyclodextrins are required for complexation of guest molecules of specific sizes. The formation of inclusion complexes leads to changes in the chemical and physical properties of the guest molecules, such as stabilization of light- or oxygen sensitive compounds, stabilization of volatile compounds, improvement of solubility, improvement of smell or tast, or modification of liquid compounds to powders. These altered characteristics of the encapsulated compounds have led to various applications of cyclodextrins in analytical chemistry (Armstrong, 1988; Loung et al., 1995), agriculture (Saenger, 1980; Oakes et al., 1991), biotechnology (Allegre and Deratani, 1994; Szejtli, 1994), pharmacy (Albers and Muller, 1995; Thompson, 1997), food (Allegre and Detrani, 1994; Bicchi et al., 1999) and cosmetics (Allegre and Detrani, 1994).

A major drawback for the application of cyclodextrins on a large scale is that all enzymes used today produce a mixture of cyclodextrins. Two different industrial approaches are used to purify the cyclodextrin mixtures: selective crystallization of beta-cyclodextrin, which is relatively poorly water-soluble, and selective complexation with organic solvents. Major disadvantages of the latter method is the toxitiy, flammability, and need for solvent recovery (Pedersen et al., 1995). This makes the production of cyclodextrins too costly for many applications. Additionally, the use of organic solvents limits applications involving human consumption.

For the industrial production of cyclodextrins, starch is first liquefied by a heat-stable alpha-amylase and then the cyclization occurs with a cyclodextrin glycosyltransferase from *Bacillus macerans* (Riisgaard, 1990) sp. A major drawback of this process is that the cyclization reaction has to be performed at lower temperatures than the initial liquefaction because of the low thermostability of the bacillus cyclodextrin glycosyltransferase. The use of cyclodextrin glycosyltransferase

from thermophilic microoganisms can solve this problem. Thermostable cyclodextrin glycosyltransferases have been found in a *Thermoanaerobacter* species (Starnes, 1990; Norman and Jørgensen, 1992; Pedersen et al., 1995), *Thermoanaero-bacterium thermosulfurogenes* (Wind et al., 1995), and *Anaerobranca bogoriae* (Prowe et al., 1996).

Cyclodextrin glycosyltransferases can also be used for the production of novel glycosylated compounds, making use of the transglycosylation activity. A commercial application is the glycosylation of the intense sweetener stevioside, isolated from the leaves of the plant *Stevia rebaudania*, thereby increasing solubility and decreasing the bitterness (Pedersen et al. 1995).

Other cyclic products that can be generated from starch are cycloamyloses. These large cyclic glucans (DP >20) contain antiparalel helices, providing long cavities with a diameter similar to that of alpha-cyclodextrin. Unlike cyclodextrins, cycloamylose is formed by all transglycosylating enzymes of the alpha-amylase family (Takata et al., 1996; Terada et al., 1997; Terada et al., 1999). Formation of cyclodextrins occurs by an intramolecular transglycosylation reaction whereas the formation of large cycloamylose molecules is the result of an intramolecular transglycosylation. To form cycloamylose, low concentrations of high molecular weight amylose are incubated with a relatively high amount of enzyme. This reaction is therefore not based on a novel catalytic mechanism but is a direct effect of the limited availability of acceptor molecules. Production of cycloamylose is currently not done on an industrial scale.

alpha-Amylase, pullulanase, cyclodextrin glycosyltransferase, and maltogenic amylase are nowadays widely used by industry in various applications (Table 3). alpha-Amylase probably has the most wide-spread use. Besides their use in hydrolysis leading to the saccharification or liquefaction of starch, these enzymes are also used for the preparation of viscous, stable starch solutions used for the warp sizing of textile fibers, the clarification of haze formed in beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility. A growing new area of application of alpha-amylases is in the fields of laundry and dish-washing detergents. A modern trend among consumers is to use colder temperatures for doing the laundry or dish-washing. At these lower temperatures the removal of starch from cloth and porcelain becomes more problematic. Detergents with alpha-amylases optimally working at moderate temperatures and alkaline pH can help to solve this problem.

Two starch-modifying enzymes of the alpha-amylase family that do not find large scale application yet are amylomaltase and branching enzyme. Application of branching enzymes is limited by the lack of commercially available enzymes that are sufficiently thermostable. A potentially interesting industrial application of amylomaltase is the production of thermoreversible starch gels. As already indicated above, a normal untreated starch gel cannot be dissolved in water after it has retrograded. However, starch that has been treated with amylomaltase has obtained thermoreversible gelling characteristics: it can be dissolved numerous times upon heating. This behaviour is very similar to gelatine. Van der Maarel et al. (2000) described this process using the amylomaltase from the hyperthermophilic bacterium *Thermus thermophilus*. Currently, no amylomaltases are commercially available and the thermoreversible starch gel is not produced on an industrial scale.

Table 1. The four conserved regions and the corresponding b-sheets found in the amino acid sequence of amylomaltase and alpha-amylase family enyzmes. Highlighted are the conserved active site amino acid residues. The following enzymes were used for the alignment: amylomaltase of *Thermus aquaticus* (Terada et al. 1999); amylosucrase of *Neisseria polysaccharea* (Büttcher et al. 1997); CGTase: cyclodextrin glucosyltranferase of *Bacillus circulans* 251 (Lawson et al. 1994); CMDase: cyclomaltodextrinase of *Clostridium thermohydrosulfuricim* 39E (Podkovyrov & Zeikus 1992); BE: branching enzyme of *Bacillus stearothermophilus* (Kiel et al. 1991); isoamylase of *Pseudomonas amyloderamosa* (Amemura et al. 1988); M. amylase: maltogenic alpha-amylase of *Bacillus stearothermophilus* (Cha et al. 1998); pullulanase of *Bacillus flavocaldarius* KP 1228 (Kashiwabara et al. 1999); sucrose Pase: sucrose phosphorylase of *Escherichia coli* K12 (Aiba et al. 1996); Taka-amylase: alpha-amylase of *Aspergillus oryzae* (Matsuura et al. 1980). b2, b4, b5, and b7 indicate the beta-sheet in which this region is present.

	Region Ib2	Region IIb4	Region IIIb5	Region IVb7
Amylomaltase	EALGIRIIG**D**MPIFVAED	LFHLV**RID**HFRG	VPVLA**E**DLGVI	VVYTGT**HD**NDT
Amylosucrase	HEAGISAVV**D**FIFNHTSN	GVDIL**RMD**AVAF	VFFKS**E**AIVHP	VNYVRS**HD**DIG
CGTase	HAKNIKVII**D**FAPNHTSP	GIDGI**RMD**AVKH	VFTFG**E**WFLGV	VTFIDN**HD**MER
CMDase	HDNGIKVIF**D**AVFNHCGY	DIDGW**RLD**VANE	AIIVG**E**VWHDA	FNLIGS**HD**TER
BE	HQAGIGVIL**D**WVPGHFCK	HVDGF**RVD**AVAN	ILMIA**E**DSTDW	FILPFS**HD**EVV
Isoamylase	HNAGIKVYM**D**VVYNHTAE	GVDGF**RFD**LASV	LDLFA**E**PWAIG	INFIDV**HD**GMT
M.amylase	HQKAIRVML**D**AVFNHSGY	DIDGW**RLD**VANE	AYILG**E**IWHDA	FNLLGS**HD**TPR
Pullulanase	HAHGVRVIL**D**GVFNHTGR	GVDGW**RLD**VPNE	AYIVG**E**IWEEA	MNLLTS**HD**TPR

Sucrose Pase LGECSHLMF**D**FVCNHMSA GAEYV**R**L**D**AVGF TVII**T**ETNVPH FNFLAS**HD**GIG

Taka-amylase HERGMYLMV**D**VVANHMGY SIDGL**R**I**D**TVKH VYCIG**E**VLDGD GTFVEN**HD**NPR

Table 2. Enzymes of the alpha-amylase family that act on glucose-containing substrates, their corresponding E.C. number, the domain organization as far as it has been described, and main substrates.

Enzyme	E.C. number	Domains	Main substrate
amylosucrase	2.4.1.4		sucrose
sucrose phosphorylase	2.4.1.7		sucrose
glucan branching enzyme	2.4.1.18	A, B, F	starch, glycogen
cyclodextrin glycosyltransferase	2.4.1.19	A, B, C, D, E	starch
amylomaltase	2.4.1.25	A, B1, B2	starch, glycogen
maltopentaose-forming amylase	3.2.1.-	A, B, I	starch
alpha-amylase	3.2.1.1	A, B, C	starch
oligo-1,6-glucosidase	3.2.1.10	A, B	amylopectin
alpha-glucosidase	3.2.1.20		starch
amylopullulanase	3.2.1.41 or	A, B, H, G, 1	pullulan
cyclomaltodextrinase	3.2.1.54	A, B	cyclodextrins
isopullulanase	3.2.1.57		pullulan
isoamylase	3.2.1.68	A, B, F, 7	amylopectin
maltotetraose-forming amylase	3.2.1.60	A, B, C, E	starch
glucodextranase	3.2.1.70		starch
trehalose-6-phosphate	3.2.1.93		trehalose
maltohexaose-forming amylase	3.2.1.98		starch
maltogenic amylase	3.2.1.133	A, B, C, D, E	starch
neopullulanase	3.2.1.135	A, B, G	pullulan
malto-oligosyl trehalose	3.2.1.141		trehalose
malto-oligosyl threhalose	5.4.99.15		maltose

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Table 3. Different fields of application of enzymes belonging to the alphalpha-amylase family

Application	Enzyme
Starch liquefaction	alpha-amylase
Starch saccharification	amyloglucosidase, pullulanase, maltogenic alpha-amylase, alpha-amylase, isoamylase
Laundry detergent and cleaners; reduction of haze formation in juices, baking, brewing, digestibility of animal feed, fiber and cotton desizing, sanitary waste treatment	alpha-amylase
Cyclodextrin production	cyclodextrin glycosyltransferase
Thermoreversible starch gels	amylomaltase
Cycloamylose	amylomaltase, branching enzyme, cyclodextrin glycosyltransferase

The invention provides an isolated or recombinant nucleic acid encoding a 4-alpha- or 6-alpha-glucanotransferase, which, in a preferred embodiment, is provided with hydrolytic activity, a or functional fragment thereof. In one embodiment, the invention provides such a nucleic acid encoding an amylomaltase, the wild types of which are generaly not known for any hydrolysing activity. 4-α-Glucanotransferase (e.g. EC 2.4.1.25, amylomaltase (AMase) or D-enzyme) forms a separate family (77) of glycosyl hydrolases. However, it is closely related to the alpha-amylase family or family 13 of glycosyl hydrolases. Unlike most members of this family of enzymes 4-α-glucanotransferase is not directly involved in starch degradation, but promotes metabolism of starch degradation products inside the cell (AMase), or is involved in starch biosynthesis (D-enzyme). Recently, however, the action of amylomaltase from *Thermus thermophilus* on starch has been described, resulting in the production of a thermoreversible gel. To investigate the enzymatic properties responsible for this action the *T. thermophilus malQ* gene has been cloned and expressed in *E. coli*, and

its sequence as here been provided, allowing purification of large amounts of enzyme, and manipulation of the gene.

In order to determine the AMase reaction specificity its action on maltooligosaccharides and soluble starch was analyzed. Although the enzyme is closely related to the α-amylase family, of the wild type enzyme no hydrolyzing activity could be detected. In the disproportionation reaction the enzyme was found to prefer longer oligosaccharides as donor substrates, while shorter oligosaccharides are efficiently used as acceptors. As observed for other amylomaltases, maltose is not cleaved off and hardly used as acceptor by the enzyme.

The complete lack of hydrolyzing activity of wild type AMase and its specificity for donor and acceptor substrates makes it a very interesting enzyme to be studied regarding reaction and product specificity.

In another embodiment, the invention provides a nucleic acid encoding a enzyme or polypeptide derived from said non-hydrolysing enzyme, now provided with hydrolysing acitivity. For example, interaction with hydrophobic amino acids, such as F366, which is highly conserved in amylomaltases, is involved in the reaction specificity of the enzyme. Hydrolyzing activity can be introduced by mutating this residue, or other hydrophobic residues such as F251 or Y54. This hydrolyzing activity has significant effects on product profiles of the enzyme, indicating the necessity of complete absence of hydrolysis for the function of the wild type enzyme (the production of longer oligosaccharides from short substrates). Now that the enzyme has been provided with hydrolysing activity, it can be used in preventing retrogradation of starch. Especially useful in such prevention is the use of a newly hydrolising enzyme as provided herein that is derived from thermostable transferase, which can be found in a thermophilic micro-organism Particulary provided is such an enzyme wherein said micro-organism comprises *Thermus thermophilus*, *Thermus aquaticus* or *Aquifex aeolicus*.

Also, the branching enzyme (BE) gene from *Aquifex aeolicus* (BE Aae) was cloned, sequenced (for he amino acid sequence see fig 4) and overexpressed in *E. coli*. The thermostable branching enzyme was purified to homogeneity, and biochemically characterized. The temperature optimum for activity was 80 ^{0}C, which is the highest optimum known for branching enzymes as compared with the other known thermostable branching enzyme from *Bacillus stearothermophilus* (BE Bst) which

has a temperature optimum of 50 °C. This higher temperature optimum is very useful in hydrolysing starch. Furthermore, BE Aae was determined to be thermostable up to 90 °C compared with approximately 60 °C for BE Bst. Branching enzymes (BE) catalyze the formation of alpha- 1,6-glucosidic linkages in two steps (pres. via cov. interm.). The first step is the cleavage of an alpha-1,4-glucosidic linkage followed by a transfer of the oligosaccharide to the 6-position of another glucose present within an alpha-1,4 glucosidic chain. This results in the branching points present in starch and glycogen. It has been shown that a lot of organisms are capable of producing starch or glycogen and express BE in order to do so. From various sources the BE has been cloned and characterized. It has been shown that BE's belong to the alpha-amylase family and that they posses the four conserved regions present within the family. A 3D model of the BE from *Aquifex aeolicus* has been designed. The crystal structure of isoamylase from *Pseudomonas* was used for modelling using the program Swiss-Pdb viewer. All amino acids that are conserved in the catalytic center within thealpha-amylase family were present in the active site of the 3D-model of BE from *Aquifex aeolicus*. The most striking feature was the present of hydrophobic residues (see fig 5) at the putative acceptor site. Alignment of branching enzymes showed that these residues are highly conserved (see fig 4) . These residues are mutated to more hydrophilic residues, for example according to the table below

Table 4 Active site residue mutagenesis of BE.

function	mutant
acceptor site	W276Q W367Q
	W385Q M387S F458S
	Y460S
donor site	Y512S
catalytic site	D311N E362Q D430N

Now that a branching enzyme has been provided with hydrolysing activity, it can be used in preventing retrogradation of starch. Especially useful in such prevention is the use of a newly hydrolising enzyme as provided herein that is derived from thermostable transferase, which can be found in a thermophilic micro-organism Particulary provided is such an enzyme wherein said micro-organism comprises

Thermus thermophilus, Thermus aquaticus or *Aquifex aeolicus.*

In overview, the invention provides modified a transferase that is derived from or has an activity of an enzyme known under EC number 2.4.1.25 or 2.4.1.18, with added hydrolysing activity. These are derivable from a nucleic acid according to the invention provided with a mutation leading to an alteration or loss of a codon originally encoding a hydrophobic amino acid located in or around a acceptor, a donor or a catalytic site extending from a TIM barrel structure of said transferase. Such mutation is preferably provided by site-directed mutagenesis, wherein said codon originally encoding a hydrophobic amino acid is altered into a codon encoding an amino acid which is substantially less hydrophobic. Preferably, the hydrophobic amino acid to be changed comprises phenylalanine, tryptophan or tyrosine, and is located at aor around the positions as indicated herein in the (beta/alpha)$_8$ or TIM barel structure of the enzyme. For example, a nucleic acid is provided wherein said change in hydrophobic amino acid is located at or around an amino acid position essentially corresponding to amino acid position 54, 251, 258 or 366 of amylomaltase of *Thermus thermophilus* HB8. Furthermore, the invention provides a vector comprising a nucleic acid according to the invention and a host cell comprising a vector or a nucleic acid according to the invention.

As said, and further explained in the detailed descriptuion herein, the invention provides a method for providing a polypeptide or fragment thereof essentially having alpha glucanotransferase acitivity but having essentially no hydrolysing activity with specific hydrolysing activity said method comprising providing a nucleic acid encoding such a transferase with a mutation leading to an alteration or loss of a codon originally encoding a hydrophobic amino acid located in or around a acceptor, a donor or a catalytic site extending from a TIM barrel structure of said transferase, and provides a polypeptide obtainable therewith.

The invention also provided use a polypeptide or fragment according to the invention in reducing retrogradation of starch, such as in reducing retrogradation of amylopectine, particularly in reducing long-term retrogradation of amylpectine. The invention provides specific enzymes provided with one of more specific amino acid modifications to obtain variants of the enzyme with hydrlolysing activity, and thus with improved product specificity, altered pH optimum, improved thermostability when strating with a thermostable enzyme as provided herein, increased specific

activity, altered cleavage pattern. An enzyme as provided herein has increased ability to reduce retrogradation of starch or staling of bread.

Also, the invention provides use such a polypeptide or fragment in hydrolysing starch, said uses for example applied in the prevention or at least temporarily avoiding of staling of bakery products such as bread, or as a replacement of acid hydrolysis in the production of starch hydrolysates. Such prevention of staling comprises use of a method for reducing retrogradation of starch comprising treating said starch with a polypeptide or fragment, such as a amylomaltase or branching enzyme provide with hydrolysing activity according to the invention. Improved quality of baked products is further obtained when the alpha-glucanotransferase (e.g. amylomaltase or branching enzyme) provided with hydrolysing activity according to the invention is used in combination with other enzymes, such as α-amylase, maltogenic amylase, cyclodextrin glycosyltransferase, β-amylase, cellulase, oxidase and/or lipase Furthermore, the invention provides a bakery ingredient comprising a polypeptide according to the invention and a bakery product such as bread comprising a polypeptide according to the invention. The invention is further explained in the detailed description provided herewith.

Figure legends

Figure 1: Overall secondary structure of the amylomaltase from *Thermus thermophilus*. The central $(b/a)_8$ barrel is shown; this barrel consists of 8 β-sheets, depicted as arrows, surrounded by 8 α-helices, depicted as spirals. The amino acid residues constituting the catalytic site extend from this barrel into the active site surrounded by subdomains B1, B2 and B3 respectively. Amino acid residues involved in binding of the donor and acceptor substrates are located in and extending from subdomain B1 and loops protruding from the $(b/a)_8$ barrel.

Figure 2: A model showing the binding of a maltoheptaose substrate in the active site of the *T. thermophilus* amylomaltase. The sugar residues are numbered according to the general subsite labeling scheme proposed for all glycosyl hydrolases by Davies et al. (Biochem. J. 1997, 321: 557-559), in which the glycosidic bond between -1 and +1 is the bond which is cleaved, and the substrate reducing end is at position +3. The positively numbered subsites constitute the acceptor binding site. The following amino acid residues are shown: (i) The catalytic residues Asp293 and Glu340; (ii) those involved in interactions with the substrate by hydrogen bonds, which are indicated by dotted lines; (iii) the aromatic amino acids involved in hydrophobic stacking interactions, being Tyr54, Trp258, Phe251, and Phe366. The model was constructed manually with the program O (Jones et al. 1991 Acta Crystallogr. D55, 849-861) on basis of the 3D structures of a porcine pancreatic a-amylase-hexasaccharide complex (Machius et al. 1996, J. Mol. Biol. 260, 409-421) and a cyclodextrin glycosyltransferase-maltononaose complex (Uitdehaag et al. 1999, Nature Struct. Biol. 6, 432-436). For clarity the model does not show the conserved catalytic site residues Tyr 59, Arg 291, His 294 and Asp 395.

Figure 3: The amino acid sequence alignment of *Aquifex aeolicus* branching enzyme (glgB Aqu) with *Pseudomonas amyloderamosa* isoamylase (isoamyla) used for constructing the 3-D model of the *Aquifex aeolicus* branching enzyme. Symbols represent the following: dots, functionally similar amino acids; *, identical amino acids; s, amino acids present in a β-sheet; amino acids present in an α-helix. b1-b8

and a1-a8 represent the alternating β-sheets and α-helices, respectively, comprising the $(\beta/\alpha)_8$ barrel.

Figure 4: Detailed overview of the active site of *Aquifex aeolicus* branching enzyme, showing the catalityc amino acid residues Asp311 (D311 cat.res.), Glu362 (E362 cat.res.), and Asp430 (D430 cat.res.) and the hydrophobic amino acid residues surrounding the catalytic site Trp276 (W276), Trp367 (W367), Trp385 (W385), Met387 (M387), Phe458 (F458), Tyr460 (Y460), and Tyr512 (Y512).

Detailed description

Kinetic analysis of amylomaltase from *Thermus thermophilus* HB8: donor and acceptor specificities

Family 77 of glycosyl hydrolases consists of a single group of enzymes; 4-α-glucanotransferases (EC 2.4.1.25, amylomaltase (AMase) or D-enzyme). AMase is found in prokaryots and promotes metabolism of starch degradation products inside the cell as shown for *Escherichia coli*. In other organisms, lacking other enzymes required for growth on oligosaccharides (p.e. maltodextrin phosphorylase), it may be involved in glycogen metabolism as suggested for *Aquifex aeolicus*. D-enzyme is found in plants and is reported to be involved in in starch metabolism. Recent studies on *Chlamydomonas rheinhardtii* show that D-enzyme is essential for biosynthesis of starch.

Sequence comparisons and 3-D structure similarities show that AMase is closely related to the á-amylase family or family 13 of glycosyl hydrolases. The a-amylase family is a very diverse group of enzymes that have the ability to modify and degrade starch. In the past, many 3D structures of enzymes from the a-amylase family have been elucidated, showing that all members share an (alpha/beta)$_8$-barrel architecture of the catalytic domain, containing a conserved active site that comprises seven amino acid residues. For this reason, it is thought that all members of the a-amylase family catalyze the same reaction cycle. This is suggested to proceed according to a two-step a-retaining mechanism. In the first step an a-glycosidic bond is cleaved in the substrate and a covalently bound enzyme-glycosyl intermediate is formed. In the second step, the leaving group is exchanged for an acceptor molecule, which is then linked via a new a-glycosidic bond to the intermediate.

Recently, amylomaltases from thermophile organisms like *Thermus aquaticus* and *Thermus thermophilus* HB8 have been isolated. These enzymes have a high thermostability, which makes them suitable for industrial applications, such as the production of large cyclic glucans and the production of thermoreversible gels from starch. A 2.0 Å 3D structure of the amylomaltase from *Thermus aquaticus* shows that the enzyme consists of a compact (alpha/beta)$_8$-barrel catalytic domain with three loop excursions that are probably responsible for part of the enzyme's specificity. In the catalytic site, 6 out of the 7 conserved residues of the a-amylase family are

present, showing the close relatedness between amylomaltase and the alpha-amylase family.

Here we describe the cloning and characterization of the *T. thermophilus* AMase. Further glycosyl hydrolase families 13 and 77 are compared regarding reaction (mechanism and) specificity.

EXPERIMENTAL PROCEDURES

Escherichia coli TOP10 was used for recombinant DNA manipulations. AMase (mutant) proteins were produced with *E. coli* BL21 (DE3).

DNA manipulations - Restriction endonucleases and DNA polymerase were purchased from Pharmacia LKB Biotechnology, Sweden, and used according to the manufacturer's instructions. DNA manipulations and calcium chloride transformation of *E. coli* strains were as described {350}.

Cloning and expression of the T. thermophilus MalQ gene - A *T. thermophilus* gene library was constructed by inserting the 4-8 kb fragments of a partial *Sau*3A digest of genomic DNA in the *Bam*HI site of pZerO. This construct was transformed to E. coli TOP10 cells and plated on LB agar plates. After replicaplating the transformants were screened for amylomaltase activity by overlaying the motherplate with 5 ml of a 0.5 % soluble starch solution, incubating for 24 h at 70°C, and staining with 4 ml Lugol solution. Positive colonies showed a shift from blue to red staining due to the disproportionation of the starch chains by amylomaltase. The DNA sequence of one of these clones was determined using the dideoxynuleotide chain termination method on a cycle sequencer (Pharmacia)

The *malQ* gene was amplified with PCR using the following primers:

Forward: GGCAGCCATATGGAGCTTCCCCGCGCTTTCGG

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The PCR product was digested with *Nde*I (CATATG) and *Bgl*II (AGATCT, overhang compatible with *Bam*HI) and ligated with either plasmid pET9c or plasmid pET15b digested with *Nde*I and *Bam*HI, resulting in pGJ6002 or pCCBmalQ, respectively. Transformation of these plasmids resulted in expression of the native enzyme (pGJ6002) or of the amylomaltase with an N-terminal His_6-tag (pCCBmalQ).

Production and purification of AMase - For the production of AMase protein *E. coli* BL21(DE3), containing the pCCBmalQ vector, was grown overnight in a 1 l

flask with 250 ml LB medium containing ampicilin.

Protein determination - Protein concentrations were determined with the Bradford method {63} using the Bio-Rad reagent and bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, CA, USA).

Enzyme assays - All assays were performed in a 25 mM sodium maleate buffer (pH 6.5) at 70 °C.

Disproportionation reaction - Disproportionation activities were determineded using the ability of AMase to release glucose from oligosaccharides. Various concentrations (upto 50 mM) of (mixtures of) oligosaccharides (G2-G7) were incubated with appropriatly diluted enzyme. For the determination of donor specificity different concentrations of maltooligosaccharides as donor and methyl-α-D-glucose as acceptor.At regular time intervals 50 ìl samples were taken and added to 200 ìl GOD-PAP reagent (Roche) to measure the amount of glucose released.

Hydrolyzing activities were measured as described earlier using 1% soluble starch (Lamers & Pleuger, Belgium) as substrate and dinitrosalicylic acid to determine the number of reducing ends {680}.

In above assays 1 U of activity is defined as the amount of enzyme required for the processing of 1 :mole of donor substrate per minute. Kinetic parameters were fitted using the computer program Sigma Plot (Jandel Scientific).

Product formation from oligosaccharides was analyzed by HPLC. For this purpose 1 ml of a 25 mM G3, G5, or G7 solution was incubated with 0.1 U AMase at 70 °C for 8 h. Samples were taken at regular time intervals and the products formed were applied to a 25 cm Econosphere-NH_2 5 micron column (Alltech Associates Inc. USA) eluted with acetonitrile/water (60/40, v/v) at a flow rate of 1 ml per min.

In the assay for the disproportionation reaction various oligosaccharides (G2-G7) were used as single (donor and acceptor) substrate. The K_M and Vmax values for the formation of glucose varied with the different oligosaccharides. The highest Vmax is observed for G4, which also shows the highest affinity. No activity on G2 was observed. Adding G3 to the G5 reaction mixture resulted in a further increase in activity (Fig. 2), whereas the addition of G2 had no effect (not shown). At high G3 concentrations a decrease in activity is observed, indicating competition between G3 and G5.

The donor specificity of AMase was further investigated using the various

oligosaccharides as donor and M-α-DG as acceptor substrates. Fig 3 shows that the addition of this monosaccharide clearly affects disproportionation activities, especially with the lower concentrations of oligosaccharide (donor) substrates. At higher M-α-DG concentrations the monosaccharide has an inhibitory effect. At lower concentrations, however, it can efficiently be used as acceptor, allowing a clearer determination of affinities of the different oligosaccharides for the donor binding site. G5, showing the lowest KM value, clearly is the best donor substrate. Combined with the above observation of the stimulating effect of G3 on disproportionation of G5, this suggests that G3 is a better acceptor substrate.

Hydrolyzing activity on soluble starch was investigated, but even overnight incubation did not result in an increase of reducing ends, thus no hydrolyzing reaction is performed by the enzyme. Furthermore the enzyme was incubated with 4-nitrophenyl-α-D-maltoheptaoside-4-6-O-ethylidene (EPS; Boehringer Mannheim) (a maltoheptasaccharide which is blocked at the non-reducing end and with a para-nitrophenyl group at its reducing end). This compound is generally used for the detection of α-amylase activity. However also with this substrate no hydrolyzing activity was observed. Furthermore it reacted very weakly when accepting oligosaccharides were added, suggesting that amylomaltase is an exo-acting enzyme, requiring the presence of a non-reducing end glucose.

The oligosaccharide formation of AMase was analyzed with HPLC (Fig. 4). With G3 as substrate the initial products were G1 and G5 (see Fig 4.a). After an initial lagfase, the production of G1 increased, while G3 decreased, however with a less significant increase of G5. Various larger oligosaccharides are produced, indicating that the initial product (G5) is used as donor and G3 is mainly used as acceptor. With G5 as substrate the predominant initial products were G3 and G7, although also considerable amounts of G1, G4, G6, and G9 were formed. In both cases little maltose is produced initially, as observed previously (T. aquaticus, potato). The final production of maltose is probably caused by the tranfer of glucose from the donor to a glucose acceptor. This supported by the early formation of G4 from G5, which indicates transfer of a glucose moyety, and by the above results with MαDG, which indicate that glucose can indeed be used as acceptor.

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oligosaccharides except maltose are efficiently used as acceptor. One of the requirements of doing this efficiently is a low hydrolyzing activity, which is extremely well met in AMase. The complete lack of hydrolyzing activity of this enzyme makes it a very interesting enzyme to be studied regarding reaction specificity in the α-amylase family.

Reaction kinetics of the disportionation of oligosaccharides

DP	Km	Vmax
2	nd	nd
3	10	90
4	3.4	281
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6	3.5	143
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Affinity constants for oligosaccharides using MαDG as acceptor

DP	0 mM	4 mM	10 mM
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STRUCTURES OF THE THERMOSTABLE AMYLOMALTASE FROM THERMUS THERMOPHILUS HB8 IN TWO DIFFERENT SPACE GROUPS

Enzymes from the alpha-amylase family, or glycosyl hydrolase family 13, are a very diverse group of starch-converting enzymes, which have a common architecture of their catalytic site. Many enzymes from the alpha-amylase family are used in industrial starch processing, and many have been structurally characterized with the aim of improving them for specific applications. Because of a lack of sufficient homology to allow Molecular Replacement, the phase problem for most of these structures has been solved by using multiple isomorphous replacement (MIR) or multi wavelength anomalous dispersion (MAD) approaches.

Here we show that the phase problem in the alpha-amylase family can be solved by using six 'high potential' heavy atom compounds that bind to conserved elements in the family. The effectiveness of this strategy was demonstrated by the elucidaton of the structure of the amylomaltase from *Thermus thermophilus* HB8, which is the most divergent member of the alpha-amylase family.

The structure of the amylomaltase from *Thermus thermophilus* HB8 was solved in space groups $P2_12_12$ and C2, whereas the highly (99.8%) identical amylomaltase from *Thermus aquaticus* was solved earlier in space group $P6_4$. A comparison of these three structures shows that the core of the enzyme is highly rigid, whereas some loops around the catalytic site can vary in conformation.

1. Introduction

The alpha-amylase family is a very diverse group of enzymes that have the ability to modify and degrade starch. Some well-known members of this family, such as bacterial alpha-amylases, cyclodextrin glycosyltransferase, and iso-amylase are used in industrial starch processing. Other enzymes, such as human salivary and pancreatic alpha-amylases are therapeutic targets in the treatment of diabetes, whereas insect alpha-amylases are useful targets in the development of crop protectants. In the past, many 3D structures of enzymes from the alpha-amylase family have been elucidated (Table 1), showing that all members share an $(alpha/beta)_8$-barrel architecture of the catalytic domain, in which a conserved active site is that comprises seven amino acid residues. For this reason, it is thought that all members of the alpha-amylase family catalyze the same reaction cycle. This is

suggested to proceed according to a two-step a-retaining mechanism. In the first step an a-glycosidic bond is cleaved in the substrate and a covalently bound enzyme-glycosyl intermediate is formed. In the second step, the leaving group is exchanged for an acceptor molecule, which is then linked via a new a-glycosidic bond to the intermediate.

The most divergent member of the alpha-amylase family is, on basis of sequence comparisons, the enzyme amylomaltase. Amylomaltase is a 57 kDa intracellular enzyme that is also known as 4a-glucanotransferase in bacteria and D-enzyme in plants. Investigations with *Escherichia coli* have established that amylomaltase is the product of the MalQ gene and is essential for the growth on maltose. Presumably, the function of the enzyme is to synthesize long amylose-like oligosaccharides from shorter oligosaccharides, which can then be further catabolized. This synthesizing capacity of amylomaltase is probably related to the enzyme's high transglycosylation activity. This forms an interesting contrast with the activity of 'classical' alpha-amylases that degrade starch and mainly perform hydrolysis.

Recently, amylomaltases from thermophile organisms like *Thermus aquaticus* and *Thermus thermophilus* HB8 have been isolated. These enzymes have a high thermostability, which makes them suitable for industrial applications, such as the production of large cyclic glucans and the production of thermoreversible gels from starch. A 2.0 Å 3D structure of the amylomaltase from *Thermus aquaticus* shows that the enzyme consists of a compact (alphalpha/betaeta)$_8$-barrel catalytic domain with three loop excursions that are responsible for part of the enzyme's specificity. In the catalytic site, 6 out of the 7 conserved residues of the alpha-amylase family are present, establishing amylomaltase as a member of the alpha-amylase family.

We have investigated two 3D structures of the amylomaltase from *Thermus thermophilus* HB8 (TTHB8), determined from data in space groups $P2_12_12$ and C2 to 2.3 Å and 3.1 Å, respectively. The TTHB8 enzyme has a 99.8% sequence identity to the amylomaltase from *Thermus aquaticus*, which crystallizes in space group $P6_4$. However, the structures of the TTHB8 enzyme were solved independently using a MIRAS strategy with general applicability for alpha-amylase enzymes.

2. Materials and methods

2.1 Crystallization and data collection

The amylomaltase from *Thermus thermophilus* HB8 was cloned and expressed in *E. coli*, and purified by a series of standard chromatographic steps until all heterogeneities had dissappeared as judged from silver-stained SDS page. The isolation and characterization of the enzyme will be described in detail in another publication. For crystallization an enzyme preparation in 25 mM Tris-HCl, pH 7.5 was used, which was concentrated to 2.5 mg/ml using a Filtron 30K system. The TTHB8 amylomaltase was crystallized at 293 K with the hanging drop vapor diffusion method, using a reservoir solution of 12% (w/v) PEG 20000 and 100 mM MES (2-[N-morpholino] ethanesulfonic acid) buffer at pH 6.8. Crystals appeared after five days, in the form of very thin plates with dimensions 0.15 x 0.15 x 0.04 mm^3. Prior to data collection, they were frozen in a cryo-mother liquor consisting of 25% (v/v) glycerol, 10% (w/v) PEG 20000 and 100 mM MES buffer at pH 6.8.

Due to the small dimensions of these crystals, the diffraction of the amylomaltase crystals on a rotating anode source was limited to 8 Å resolution. However, by using synchrotron radiation, a complete dataset could be obtained to 2.3 Å resolution. The intensity distribution of the data was very anisotropic, most likely because of the non-uniform dimensions of the crystals. The space group of the crystals was $P2_12_12$, with cell dimensions a=115.2 Å, b=93.7 Å, c=53.5 Å.

In addition to this crystal form, small microneedles (0.04 x 0.04 x 0.20 mm^3) were found growing in hanging drops at 12% (w/v) PEG 20000 and 100 mM maleate at pH 6.8 and 0.1% (w/v) maltotriose. These crystals were frozen by transferring them to an identical solution to which 20% (v/v) glycerol was added, and subsequently dipping them in liquid nitrogen. The frozen crystals were exposed to synchrotron radiation and belong to space group C2, with cell dimensions a=104.9 Å, b=52.5 Å, c=104.9 Å, and a=90°,b= 96.4°, and g=90°. Unfortunately, their diffraction was limited to 3.1 Å, therefore we performed further soaking experiments with the better-diffracting $P2_12_12$ crystal form.

2.2 Phasing

Because sequence comparisons suggest that amylomaltase is a member of the alpha-amylase family, we initially attempted to solve the phase problem for

amylomaltase by Molecular Replacement using a poly-alanine TIM barrel domain as search model. Several models were tried, originating from cyclodextrin glycosyltransferase (CGTase) and *Aspergillus oryzae* (Taka) alpha-amylase, but all attempts failed. This is not surprising since similar Molecular Replacement attempts were also problematic in cases in the alpha-amylase family where model and target had much more structural homology. Therefore, as a next strategy, we decided to use ab initio phasing with multiple isomorphous replacement combined with anomalous scattering (MIRAS).

To determine a suitable MIRAS strategy, an overview was made of the compounds that were used in the past to solve structures of enzymes from the alpha-amylase family. It appears that many structures have been solved using the same heavy atom compounds. Out of 14 cases, a $HgCl_2$ derivative was useful 8 times, a K_2PtCl_4 derivative 7 times, a UO_2^{2-} derivative 6 times and a Sm^{3+} derivative 3 times. This suggests that these compounds bind to conserved features in alpha-amylase family enzymes and thus would have general applicability within the family.

To check this hypothesis and to solve the structure of the TTHB8 amylomaltase, we used these compounds to soak crystals and collected data at the EMBL beamline BW7B at DESY, Hamburg and the EMBL beamline ID14-3 of the ESRF, Grenoble (Table 3). Despite non-isomorphism in the length of the longest cell axis, all the four above-mentioned compounds turned out to be useful derivatives. In addition we found an ethylmercury phosphate derivative.

From these data, heavy atom sites were located using the program Solve and subsequently refined with the program Sharp. Solvent flattening resulted in an experimental electron density map in which secondary structure elements were well discernible. Model building was was performed with the program O. To facilitate model building, we used the sequence and structure of amylomaltase from *Thermus aquaticus* as a template.

2.3 Refinement of the $P2_12_12$ crystal form

Our initial model was refined against our best data, those from a $HgCl_2$ soak which diffracted to 2.3 Å. Refinement was performed using the program CNS version 1.0. After initial rigid body refinement, full coordinate refinement, grouped B-factor refinement and individual atomic B-factor refinement against the CNS maximum likelihood target were applied. Solvent molecules were placed at peaks of at least 3.0

s in F_o-F_c difference electron density maps, at positions where they could form at least one hydrogen bond. This was done using in combination with refinement using the iterative procedure implemented in CNS. Manual rebuilding was done in s_A-weighted F_o-F_c, $2F_o$-F_c and OMIT F_o-F_c,and $2F_o$-F_c maps, calculated with CNS.

During rebuilding, a very strong peak close to a small peak in an F_o-F_c difference electron density was observed in the active site. A peak in a similar position was observed in an anomalous difference map (F_o^+-F_o^-, where + and - reflections are Bijvoet mates) from the $HgCl_2$ data. Moreover, the program Sharp had interpreted the position of this peak as a heavy atom binding site. From this we concluded that a $HgCl^-$ ion bound in the active site should be included in our model.

The stereochemistry of the final model was checked with the programs Procheck and Whatcheck. The final model contains no residues in disallowed regions of the Ramachandran plot, in contrast to the structure of the *Thermus aquaticus* enzyme. The atomic coordinates and structure factors have been deposited at the Protein Data Bank (www.rcsb.org, code 1FP8).

2.4 Refinement of the C2 crystal form

In order to study the influence of crystal contacts on the conformation of the enzyme, we also determined the 3.1 Å structure of TTHB8 amylomaltase in the maltotriose-dependent C2 crystal form. An initial model was obtained from the structure in $P2_12_12$, by Molecular Replacement with the program AMoRe. This model was refined using CNS as outlined above. The final refinement step consisted of a few rounds of individual B-factor refinement, which was stopped after the free R-factor started to increase. No solvent molecules were incorporated. Although the crystals were grown in the presence of sugars, we found no evidence for the presence of maltotriose or any other oligosaccharide in the electron density maps. Final model statistics, coordinates and structure factors have been deposited at the Protein Data Bank (www.rcsb.org, code 1FP9).

2.5 Binding locations of heavy atom ligands

To solve the phase problem for amylomaltase we used heavy atom compound with a high success rate in the alpha-amylase family, under the assumption that they bind to conserved features within the family. To check whether this is true, we investigated their location using anomalous difference electron density maps

computed with phases from refined models. As indicated above, the $HgCl_2$ soak resulted in a $HgCl^-$ ion bound in the conserved catalytic site of the alpha-amylase family. At that position, the Cl^- atom binds to Tyr 59 with a typical halide-aryl interaction, whereas the Hg^{2+} atom is bound by the conserved acidic residues Asp 395, Glu 340 and Asp 293.

In addition to $HgCl_2$, the ethylmercury phosphate soak also resulted in an active site complex in which an Hg^{2+} moiety is bound by acidic residues. However, the other soaks (UO_2Ac_2, K_2PtCl_4, $SmCl_3$) resulted in heavy atoms bound in non-conserved regions (Table 3). This contrasts with other reports. UO_2Ac_2 was observed to bind in the active site of the CGTase from *Bacillus circulans* strain 251, and $UO_2(NO_3)_2$ was observed in the active site of Taka alpha-amylase. K_2PtCl_4 was observed to bind close to the catalytic site in the CGTase from *Bacillus circulans* strain 8, near residue His 233 in the sugar binding subsite +1. In Taka alpha-amylase, K_2PtCl_4 was observed to bind close to the catalytic site. To explain this discrepancy, we suggest that the binding of UO_2Ac_2 and K_2PtCl_4 in the active site of amylomaltase is hindered by the presence of a low concentration of $HgCl_2$ that was applied to stabilize the crystals. The $HgCl_2$ might compete with the other compounds for binding.

Thus, we show that there exist 'high potential' compounds, which are much more succesful than average in forming heavy atom derivatives of a crystallized alpha-amylase family enzymes. Most of these compounds were reported to bind in the conserved catalytic site, though this could not always be reproduced for TTHB8 amylomaltase. Nevertheless, it was shown that with these compounds, the phase problem for alpha-amylase-family enzymes can be quickly and efficiently solved.

3. Results

3.1 Secondary structure

The three-dimensional structure of the amylomaltase from *Thermus thermophilus* HB8 in its $P2_12_12$ crystal form is depicted in Figure 1. It is similar to the *Thermus aquaticus* amylomaltase and consists of a central $(alpha/beta)_8$ or TIM-barrel domain from which three other small domains protrude. Although the $(alpha/beta)_8$-barrel domain is a feature that is shared by all enzymes from the alpha-amylase, a superposition of the $(alpha/beta)_8$-barrel domain in amylomaltase with those from

cyclodextrin glycosyltransferase (CGTase) and Taka alpha-amylase shows large differences in the position, length and orientation of the a-helices that surround the central b-barrel. These differences explain the difficulty of solving the phase problem by using TIM-barrels from alpha-amylase family enzymes as templates for a Molecular Replacement search. Moreover, they shows that the folding pattern of the (alpha/beta)$_8$-barrel is more conserved than the precise three-dimensional orientation of its constituent secondary structure elements.

From the central (alpha/beta)$_8$-barrel domain in amylomaltase three subdomains protrude that are labelled B1, B2 and B3. Subdomain B2 comprises residues 68 to 179 and protrudes at the third beta-strand of the TIM barrel, which makes this subdomain the structural homolog of domain B in CGTases and alpha-amylases. Subdomain B1 comprises residues 222 to 272 and 294 to 320, and subdomain B3 comprises residues 398 to 427. Both these latter domains are unique to amylomaltase.

3.2 The active site of amylomaltase

Another determinant of alpha-amylase family membership is the presence of seven conserved residues in the catalytic site in a characteristic orientation. The catalytic site of the TTHB8 amylomaltase is compared with the catalytic site of CGTase, a representative member of the alpha-amylase family. It appears that the nucleophilic catalytic residue Asp 229 in CGTase, and the acid/base catalyst Glu 257 have amylomaltase equivalents in Asp 293 and Glu 340. Residues Arg 227, His 327 and Tyr 100, which are important in strabilization of the transition state and the covalent intermediate have equivalents in amylomaltase in Arg 291, His 394 and Tyr 59, respectively. Interestingly however, of two residues in CGTase that are important for distortion of a bound substrate, Asp 328 and His 140, only Asp 328 has an equivalent in amylomaltase in Asp 293, whereas the position of His 140 is taken by Asn 260. In this respect, amylomaltase is different from all other members of the alpha-amylase family. Interestingly, when His 140 is replaced in CGTase or alpha-amylase, the activity decreases 50-100x times.However, amylomaltase has an optimal enzymatic rate that is comparable to that of other alpha-amylases. This might indicate that amylomaltase has found a way of compensating for the absence of a His 140 equivalent by an unknown mechanism.

3.3 Putative sugar binding sites

In addition to the catalytic site, amylomaltase possesses at least seven sugar binding subsites that assist in substrate processing. We attempted to identify these sugar binding subsites by a crystal-soaking procedure, in which $P2_12_12$ crystals of TTHB8 amylomaltase were subjected to a stabilizing solution containing the oligosaccharide inhibitor acarbose. This inhibitor is known to bind strongly in the catalytic site of alpha-amylase-family enzymes and in adjacent sugar binding subsites. Unfortunately, after subsequent data collection on these crystals, this inhibitor could not be observed in the electron density, and therefore had not bound inside the crystals. Probably, the active site of amylomaltase in its $P2_12_12$-crystalline form is not accessible to oligosaccharide binding.

To nevertheless estimate the location of extra sugar binding subsites, we constructed a model of sugar binding. We superimposed the 3D structure of a maltohexaose inhibitor in complex with Porcine pancreatic alpha-amylase on amylomaltase on basis of the conserved active site in both enzymes. The torsion angles of the glycosidic bonds in the maltohexaose inhibitor were subsequently adjusted to improve its fit in the active site of amylomaltase. This remodelling was aided by comparisons with the conformations of other oligo-saccharides in complex with alpha-amylase family enzymes, such as maltononaose bound to CGTase. The final model is schematically drawn in Figure 3, and is the first detailed model of how amylomaltase might bind an oligosaccharide, and is provides the guidance needed for site-directed mutagenesis experiments that alter the properties of amylomaltase in a desired fashion.

3.4 Crystal contacts in the $P2_12_12$ and C2 crystal forms

The structure of TTHB8 amylomaltase was determined to high resolution in a $P2_12_12$ crystal form, and to lower resolution in a C2 crystal form. This allows us to establish whether the conformation of amylomaltase is influenced by the crystalline packing of the molecules.

In the $P2_12_12$ form, crystal contacts are formed in three regions. In the first a loop of residues Gly 149-Gly 153 is grabbed by residues Gly 422-Arg 426 and the C-terminus Ala 492-Leu 500. A second, weaker contact is formed between residues Gln 27-Glu 38 and the two stretches Glu 313-:Lys 318, Gly 343-Val 349.

Interestingly, a third contact is formed by the only cysteine residue in amylomaltase. Cys 308 is at a distance of 5.2 Å of a Cys 308 of another amylomaltase molecule inside the crystal. This suggests that one crystal contact is formed by an intramolecular disulphide bond. The possibility of a disulphide-linked crystal contact is corroborated by the electron density at this location, which suggest the (partial) presence of a disulphide bond. This suggests that the crystal lattice consists of a mixture of disulphide-bonded dimers and monomeric units. Dynamic light scattering experiments with our sample support the presence of a small amount of dimers (results not shown) mixed with monomers. It is not unlikely that the dimeric impurities enforce the presence of a disulphide-bonded crystal contact, inhibiting the formation of other (stronger) contacts, such as present for example in the $P6_4$ crystal form of the *Thermus aquaticus* amylomaltase.

The crystals in space group C2 show a similar disulphide-linked crystal contact. However, at the other crystal contacts, there are significant differences. The stretch of residues Lys 148-Glu 173 binds to Pro 378-Gly 385, and the residues Gly 26-Asp 31 and Leu 74- Gly 89 bind to Gly 26-Asp 31 and Leu 74- Gly 89 in another molecule. Due to these differences, the C2 crystal form can be regarded as independent from the $P2_12_12$ crystal form.

3.5 Comparison of amylomaltases from *T. aquaticus* and *T. thermophilus* HB8

We have determined two structures of the amylomaltase from *Thermus thermophilus* HB8. Earlier, the 3D structure of the amylomaltase from *Thermus aquaticus* was determined, which has a sequence identity to the TTHB8 amylomaltase of 99.8%. Only Gln 27 and Leu 154 in the TTHB8 enzyme have been substituted by Arg 27 and Pro 154 in the *Thermus aquaticus* enzyme. Strangely, the published amino acid sequence of the *Thermus aquaticus* enzyme, which gives Pro 154, does not correspond to the sequence derived from the 3D structure of the *Thermus aquaticus* amylomaltase, which gives Leu 154. If this is interpreted as a correction on a sequencing error, both amylomaltases only differ in amino acid sequence at position 27. Therefore, for all practical purposes these structures can be regarded as independently solved structures of the same enzyme in different space groups. A comparison could reveal interesting areas of flexibility.

3.6 Conformational differences between the three structures of amylomaltase

Since the structures of *T. thermophilus* HB8 amylomaltase in space groups C2 and $P2_12_12$ and the structure of *T. aquaticus* amylomaltase in space group $P6_4$ can be regarded as three structures of the same enzyme in different crystal packing environments, differences between these structures can show how crystal contacts influence the conformation of the enzyme, and in which areas it is very flexible or very rigid.

If we take the '$P2_12_12$-structure' as basis, and superimpose the 'C2-structure', we observe that the position of most amino-acids is identical (r.m.s.d. 0.5 Å). However, two loops in the active site cleft have a significantly different conformation. First, the loop that comprises residues Tyr 141 to Ala 170 has shifted in the C2-form towards the active site (maximally 1.5 Å). Secondly, the loop of residues Val 242-Leu 262 (and its adjacent loop Tyr 301-Val 317), which cover the active site cleft, have shifted ~0.5 Å outwards in the C2 form, thereby opening the cleft a little.

When the structure of *T. aquaticus* amylomaltase is superimposed on the '$P2_12_12$-structure', this shows that they have an almost identical conformation (r.m.s.d. 0.4 Å). Interestingly, also in the *T. aquaticus* enzyme the loop of residues 141-170 has a position that is oriented more toward the active site (maximum difference 1.3 Å). This position resembles the conformation of this loop in the C2-crystal form.

The flexibility of amylomaltase was further studied through the atomic temperature factors. In general, all three structures show a similar temperature factor distribution, indicating only a marginal influence of crystal packing contacts. In all cases amylomaltase appears to be rigid, with specific areas having higher temperature factors, and thus higher flexibility. These include four loop stretches near the catalytic site comprising residues 80-93, 114-125, 342-348 and most strongly 249-253.

Thus, in general amylomaltase appears to have a rigid, well-determined conformation, which might in part explain the enzyme's thermostability. However, when information on conformational variability and temperature factor distributions is combined, it appears that there are two interesting regions in the enzyme. The first is the loop 242-262 (comprising 249-253) that can have different conformations and is also very flexible (high B-factors). This loop incorporates residues Tyr 250 and Phe 251, which might be involved in substrate

binding (Figure 3). The second is the loop 141-170, which is conformationally variable but has a very low temperature factor. Therefore, this loop is not flexible, but can 'switch' between two rigid conformations. As was observed for other alpha-amylase family enzymes, such conformational variations could play an important role in promoting catalysis.

Overview of heavy atom compounds used to solve 3D structures of alpha-amylase-family proteins

Enzyme	method[c]	heavy atom compounds used
Animal alpha-amylases		
porcine pancreas	MIR (2x)[a]	OCMP[b]/K_2PtCl_4/K_2HgI_4/$PbNO_3$/$HgAc_2$/U_2O_7
human salivary	MR	
human pancreas	MR	
yellow meal worm	MR	
Fungal and plant alpha-amylases		
Taka (*Aspergillus oryzae*)	MIR	$HgCl_2$/$UO_2(NO_3)_2$/$AgNO_3$/K_2PdCl_4/K_2PtCl_4/$K_2Pt(CN)_4$/$KAu(CN)_2$
Aspergillus niger	MIR/MR	$HgCl_2$/$SmAc_3$/K_2PtCl_6/$PbAc_2$
Barley	MIR	$HgCl_2$/$Eu(NO_3)_3$/K_2PtCl_4
bacterial alpha-amylases		
B. licheniformis	MIR (2x)	UO_2Ac_2/$Pb(CH_3)_3Ac$/$HgCl_2$/K_2PtCl_4/K_2PtCl_6[a]
B. subtilis	MIR	K_2PtCl_4/$HgCl_2$
P. stutzeri	MIR	$K_3UO_2F_5$/$SmCl_3$
Alteromonas haloplanctis	MR	
cyclodextrin glycosyltransferases		
B. circulans strain 8	MIR	K_2PtCl_4/cis-$(NH_3)_2PtCl_2$/$UO_2C_2O_4$
B. stearothermophilus		
B. sp. 1011	MR	
B. circulans strain 251	SIRAS	UO_2Ac_2
other enzymes		
B. cereus oligo-1,6-glucosidase		$HgCl_2$/$UO_2(NO_3)_2$/$Sm(NO_3)_3$
P. amyloderamosa iso-amylase	MIR	$NaAuCl_4$/$HgCl_2$
B. stearothermophilus	MR	
maltogenic alpha-amylase		
Thermoactinomyces vulgaris	MIRAS	$PbAc_2$/$C_2H_5Hg^-$
A47		
alpha-amylase II		
Thermus strain maltogenic	MIR/MR	Se-Met/PtCl2$(NH_3)_2$/$HoCl_2$
alpha-amylase		
Thermus aquaticus	MIR	PCMBS/$HgCl_2$/K_2PtCl_4/$KAu(CN)_2$/$K_2Pt(SCN)_6$/$Pb(CH_3)_3Ac$
amylomaltase		

[a]used in the most recent report. [b]OCMP means ortho-chloromercuriphenol. PCMBS means para-chloromercuriphenylsulfonic acid. [c]MIR(AS) means Multiple isomorphous replacement (with anomalous scattering), MR means Molecular Replacement, SIR(AS) means single isomorphous replacement (with anomalous scattering).

Data collection and refinement statistics

Data collection	Native $P2_12_12$	Native C2
X-ray source	EMBL beamline ID14-3 ESRF, Grenoble	
Temperature (K)	120	120
Wavelength (Å)	0.933	0.931
Space group	$P2_12_12$	C2
Cell axis (Å)	115.2, 93.7, 53.5	104.9, 52.4, 104.9
	90.0, 90.0, 90.0	90.0, 96.4, 90.0
Resolution (Å)	53.4-2.30	52.2-3.13
No. of unique reflections	26405	10132
Completeness (%)	99.7	96.3
R_{merge}[a] and <I/s>	0.076 / 16.2	0.082 / 8.7
Statistics of the last resolution shell	(2.37 Å-2.30 Å)	(3.17 Å-3.10 Å)
Completeness (%)	99.5	96.3
R_{merge} and <I/s>	0.37 / 3.5	0.20 / 2.8
Refinement statistics		
No. of amino acids	500 (all)	500 (all)
No. of solvent sites	270	0
Average B-factor ($Å^2$)	35.6	34.5
Final R-factor[b] (incl. bulk solvent correction)	0.194	0.239
Final free R-factor[c] (incl. bulk solvent correction)	0.232	0.292
R.m.s. deviation from ideal geometry		
bond lengths (Å)	0.006	0.004

bond angles (deg.)	1.204	1.031
B-factor correlations between neighbouring main chain atoms (Å^2)	1.37	2.46
percentage of residues in allowed regions of the Ramachandran plot	100.0	100.0

[a]$R_{merge}=S_hS_i|I(h)-I_i(h)|/S_hS_i\ I_i(h)$ where reflection h has intensity $I_i(h)$ on occurrence i and mean intensity I(h). [b]R factor=$S_h|F_o-F_c|/S_hF_o$ where F_o and F_c are the observed and calculated structure factor amplitudes of reflection h, respectively. [c]The free R factor is calculated as the R factor, using F_o that were excluded from the refinement (5% of the data).

Summary of phasing statistics

	unsoaked	$HgCl_2$[a]	UO_2Ac_2[b]	$(C_2H_5Hg)_3PO_4$	$SmCl_3$[b]	K_2PtCl_4
X-ray source	BW7B	ID14-3	BW7B	BW7B	BW7B	BW7B
Soaking conditions (days/mM)	-	3/3	3/3	3/5	3/10	3/3
Temperature (K)	100	120	100	100	100	100
Wavelength (Å)	0.8439	0.9330	0.8439	0.8439	0.8439	0.8439
Resolution (Å)	36.7-3.0	53.4-2.30	50-2.57	50-2.73	50-2.94	50-3.0
No. of unique reflections	11853	26436	19162	16205	13139	12372
Longest cell axis (Å) (in $P2_12_12$)	117.8	115.2	116.3	116.3	116.0	117.6
Completeness	95.0	99.7	98.2[e]	99.0[e]	98.6	99.6[e]
R_{merge} and <I/s>	0.22/5.0	0.076/16.2	0.11/12.1[e]	0.13/8.4[e]	0.14/9.1	0.15/7.6[e]
Phasing to 3.3 Å (centric/acentric)						
Number of sites	-	1	3	4	3	5
PP[c]	-	1.1/1.1	1.7/2.3	1.2/1.4	0.76/0.80	0.95/0.92
Anomalous PP[c]	-	-/-	-/1.2	-/1.0	-/-	-/0.92
Nearest amino acid residue	-	Asp 293 Asp 395 Glu 340	Asp 293[b] Asp 52 Tyr 54	His 228 His 304 His 409 Glu 460	Asp 96 Asp 369 Glu 122	Met 145 Met 372 His 409 Arg 209 Arg 194
Combined FOM[d]	0.61/0.53					

[a]Due to their high resolution, the final model was refined against these data.

[b]Because $HgCl_2$ appeared to stabilize the crystals, soakings with this compound was preceded by a soaking in $HgCl_2$. The site labelled 'b' is therefore probably a Hg^{2+} ion.

[c]PP: Phasing Power. [d]Before solvent flattening. [e]Regarding Bijvoet mates as separate reflections.

Generation of mutants

Family 77 of glycosyl hydrolases consists of a single group of enzymes; 4-α-glucanotransferases (EC 2.4.1.25, amylomaltase (AMase) or D-enzyme). AMase is found in prokaryots and promotes metabolism of starch degradation products inside the cell as shown for *Escherichia coli*. In other organisms, lacking other enzymes required for growth on oligosaccharides (p.e. maltodextrin phosphorylase), it may be involved in glycogen metabolism as suggested for *Aquifex aeolicus*. D-enzyme is found in plants and is reported to be involved in in starch metabolism. Recent studies on *Chlamydomonas rheinhardtii* show that D-enzyme is essential for biosynthesis of starch. In each case the role of AMase is based on its transglycosylating activity, which enables the enzyme to produce long oligosaccharides from short chained substrates or transfer oligosaccharides to branched polymers (glycogen, amylopectin). The synthesizing capacity of wid type amylomaltase is probably related to the enzyme's high transglycosylation activity and lack of hydrolyzing activity. This forms an interesting contrast with the activity of 'classical' a-amylases that degrade starch and mainly perform hydrolysis.

Sequence comparisons and 3-D structure similarities show that AMase is closely related to the alpha-amylase family or family 13 of glycosyl hydrolases. The a-amylase family is a very diverse group of enzymes that have the ability to modify and degrade starch. In the past, many 3D structures of enzymes from the a-amylase family have been elucidated, showing that all members share an (alpha/beta)$_8$-barrel architecture of the catalytic domain, containing a conserved active site that comprises seven amino acid residues. For this reason, it is thought that all members of the a-amylase family catalyze the same reaction cycle. This is suggested to proceed according to a two-step a-retaining mechanism. In the first step an a-glycosidic bond is cleaved in the substrate and a covalently bound enzyme-glycosyl intermediate is formed. In the second step, the leaving group is exchanged for an acceptor molecule, which is then linked via a new a-glycosidic bond to the intermediate.

Recently, amylomaltases from thermophile organisms like *Thermus aquaticus* and *Thermus thermophilus* HB8 have been isolated. These enzymes have a high thermostability, which makes them suitable for industrial applications, such as the production of large cyclic glucans and the production of thermoreversible gels from starch. A 2.0 Å 3D structure of the amylomaltase from *Thermus aquaticus* shows that

the enzyme consists of a compact (alpha/beta)$_8$-barrel catalytic domain with three loop excursions that are probably responsible for part of the enzyme's specificity. In the catalytic site, 6 out of the 7 conserved residues of the a-amylase family are present, showing the close relatedness between amylomaltase and the a-amylase family.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids - *Escherichia coli* TOP10 (Invitrogen) was used for recombinant DNA manipulations. AMase (mutant) proteins were produced with *E. coli* BL21(DE3) (Stratagene). The *malQ* gene was amplified with PCR using the following primers:

Thermus thermophilus:

Forward: GGCAGCCATATGGAGCTTCCCCGCGCTTTCGG

Reverse: GCAGCCAGATCTAGAGCCGTTCCGTGGCCTCGGC

Aquifex aeolicus:

Forward: GGCAGCCATATGAGATTGGCAGGTATTTTAC

Reverse: GCAGCCGGATCCTTAAACTTCTCTTCCG

The PCR product was digested with *Nde*I (CATATG), and *Bgl*II (AGATCT, overhang compatible with *Bam*HI, *T. thermophilus*) or *Bam*HI (GGATCC, *A. aeolicus*) and ligated with plasmid pET15b (Novagen), digested with *Nde*I and *Bam*HI. The resulting construct (pCCBmalQ) encodes the amylomaltase with an N-terminal His$_6$-tag.

Site-directed mutagenesis - For site-directed mutagenesis a method based upon PCR reactions using PWO-DNA polymerase was used. In a first PCR reaction a mutagenesis primer together with the reverse primer was used. The product of this reaction was used as a primer in a second PCR reaction together with the forward primer. This PCR product was cloned in pET15b using the same strategy as for the wild type. The following mutagenesis primers were used to produce the mutations:

Thermus thermophilus:

F251L/S: 5'-CCC'CCC'GAC'TAC'TYG'AGC'GAG'ACC'GGT'CAG'CGC'TGG' GGC-3',

F366L/S: 5'-AAG'GTC'CTG'CAA'TYG'GCC'TTT'GAC'GAC-3'

Aquifex aeolicus:

F244L/S: 5'-CCT'CCT'GAT'TTC'TYG'AGT'AAA'ACG'GG-3'

F359L/S:5'-GTT'ATT'GAG'TYG'GCC'TTC'TAC'G-3'

In these primers Y= T (F-L) of C (F-S). Successful mutagenesis resulted in appearance of the underlined restriction sites, allowing rapid screening of potential mutants. For F251L/S this restriction site was *Age*I (ACCGGT); for F251S an additional *Xho*I site (CTCGAG) was introduced. Mutation F366L/S caused deletion of a *Pst*I site; for F366L an additional *Mun*I site (CAATTG) was introduced. For F244S an *Xho*I site (CTCGAG) was introduced. All mutations were confirmed by restriction analysis and DNA sequencing.

DNA manipulations - Restriction endonucleases were purchased from Pharmacia LKB Biotechnology, Sweden; NEB; or Boehringer, and used according to the manufacturer's instructions. DNA manipulations and calcium chloride transformation of *E. coli* strains were as described.

Growth conditions - Plasmid carrying bacterial strains were grown on LB medium containing 50 ìg/ml ampicilin (*E. coli* TOP10) or 50 ìg/ml ampicilin and 50 ìg/ml chloramphenicol (*E. coli* DE3(RP)). For the production of (mutant) AMase proteins *E. coli* DE3(RP), containing the pCCBmalQ vector, was grown in a 1 l flask with 250 ml LB medium containing 50 ìg/ml ampicilin.

Protein determination - Protein concentrations were determined with the Bradford method {63} using the Bio-Rad reagent and bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, CA, USA).

Enzyme assays - All assays were performed in a 25 mM sodium maleate buffer (pH 6.5) at 70 °C.

Disproportionation reaction - Disproportionation activities were determineded using the ability of AMase to release glucose from oligosaccharides. Various concentrations (upto 50 mM) of (mixtures of) oligosaccharides (G2-G7) were incubated with appropriatly diluted enzyme. For the determination of donor specificity different concentrations of maltooligosaccharides as donor and methyl-α-D-glucose as acceptor.At regular time intervals 50 ìl samples were taken and added to 200 microliter GOD-PAP reagent (Roche) to measure the amount of glucose released.

Hydrolyzing activities were measured as described earlier using 1% soluble starch (Lamers & Pleuger, Belgium) as substrate and dinitrosalicylic acid to determine the number of reducing ends .

In above assays 1 U of activity is defined as the amount of enzyme required for the processing of 1 mmole of donor substrate per minute. Kinetic parameters were fitted using the computer program Sigma Plot (Jandel Scientific).

Product formation from oligosaccharides was analyzed by HPLC. For this purpose 1 ml of a 25 mM G3, G5, or G7 solution was incubated with 0.1 U AMase at 70 °C for 8 h. Samples were taken at regular time intervals and the products formed were applied to a 25 cm Econosphere-NH_2 5 micron column (Alltech Associates Inc. USA) eluted with acetonitrile/water (60/40, v/v) at a flow rate of 1 ml per min.

Sequence alignments - Sequence alignments of various amylomaltases indicate that the two phenylalanines selected based on the structure of amylomaltase and the model of the maltoheptaose bound in the active site are (functionally) conserved in all amylomaltases.

T.thermophilus GVPPDYFSETGQRWGNP
T.aquaticus {4} GVPPDYFSETGQRWGNP
Synechocystis {101} GVPPDYFSATGQLWGNP
A.aeolicus {97} GVPPDFFSKTGQLWGNP
S.tuberosum {25} GVPPDAFSETGQLWGSP
C.butyricum {11} GCPPDAFSETGQLWGNP
S.pneumoniae {55} GCPPDEFSVTGQLWGNP
M.tuberculosis {96} GAPPDEFNQLGQDWSQP
H.influenzae {103} GAPPDPLGPVGQNWNLP
E.coli {40} GAPPDILGPLGQNWGLP
C.pneumoniae {94} GAPPDLYNSEGQNWHLP
C.psittaci {99} GAPPDIYNTEGQNWHLP
C.trachomatis {95} GAPPDLYNAEGQNWHLP

T.thermophilus LAEDLGVITPEVEALRDRFGLPGMKVLQFAF
T.aquaticus LAEDLGVITPEVEALRDRFGLPGMKVLQFAF
Synechocystis VAEDLGVITPEVEALRDEFNFPGMKVLHFAF
A.aeolicus IAEDLGFITDEVRYLRETFKIPGSRVIEFAF
S.tuberosum IAEDLGVITEDVVQLRKSIEAPGMAVLQFAF
C.butyricum IAEDLGYLTEETLEFKKRTGFPGMKIIQFAF
S.pneumoniae IAEDLGFMTDEVIELRERTGFPGMKILQFAF
M.tuberculosis VGEDLGTVEPWVRDYLLLRGLLGTSILWFEQ
H.influenzae IGEDLGTVPDEVRWKLNEFQIFSYFVLYFAQ
E.coli IGEDLGTVPVEIVGKLRSSGVYSYKVLYFEN
C.pneumoniae IGEDLGIIPQDVKTTLTHLGICGTRIPRWER
C.psittaci IGEDLGSVPTDVKETLVKLGICGTRIPRWER
C.trachomatis IGEDLGTIPSDVKRMLESFAVCGTRIPRWER

Construction of mutant enzymes - One mutant (F366L, *Thermus*) has been constructed and confirmed by sequence analysis. Other mutants have been constructed (for example F366S (*Thermus*), F359L/S (*Aquifex*))

Disproportionation activity - Mutant F366L has been analyzed concerning the disproportionation of maltotriose. The activity (25 U/mg) was four times lower than that of the wild type, whereas the affinity (Km = 3.5) was threefold higher than the wild type.

Hydrolyzing activity - As for the wild type, no hydrolyzing activity could be determined during incubation of soluble starch, even with large amounts of enzyme. However, contrary to the wild type enzyme, an increase in reducing power of the reaction mixture after overnight incubation was detected, indicating that hydrolysis had taken place.

Product formation from maltotriose - HPLC analysis of the products formed during incubation of the enzyme with maltotriose clearly shows that hydrolysis takes place. Whereas the wild type produces essentially no maltose, which cannot be cleaved of by the enzyme, the mutant produces maltose as one of the main compounds.

Interaction with hydrophobic amino acids, such as F366, which is highly conserved in amylomaltases, is involved in the reaction specificity of the enzyme. Hydrolyzing activity can be introduced by mutating this residue or other hydrophobic residues. This hydrolyzing activity has significant effects on product profiles of the enzyme, indicating the necessity of essentially complete or practically complete absence of hydrolysis for the function of the wild type enzyme (the production of longer oligosaccharides from short substrates).

Alignement of Branching Enzymes

Bstearothermophilus ----------
Bcaldolyticus ----------
Bsubtilis ----------
mycobacterium ----------MSRSEKLTGEH-LAPEPA----
EMARLVAGT
Streptomyces
MSAARQPSPTVRDKAAPEPAAPAAPKGARAPRARRAAPPHGVRPAPALAAEERAR
LLEGR
E. ----------MSDRIDRDVINALIAGH
H.influenzae ----------MTTAVTQAIIDGFFDAS
Agro.tume ----------MKKPLNSAEEKKTGDITKAEIEAIKSGL
Aquifex_a. ----------
Synechococcus ----------TGTTPLPSSSLSVEQVNRIASNQ
Synechocystis ----------MTYTINADQVHQIVHNL
Butyrivibrio ----------
CHLAMYDIA ----------MDPFFLNTQHVELLVSGK

Bstearothermophilus ----------
Bcaldolyticus ----------
Bsubtilis ----------
mycobacterium HHNPHGILGAHEYDDHTVIR-----AFRPHAVEVVALVGK---
DRFSLQHLD-SGLFAVA
Streptomyces HHDPHAVLGARTQRGGVAFR-----VLRPYAKAVTVVAKG---
LRTELVDEG-DGLFSGL
E. FADPFSVLGMHKTTAGLEVR-----
ALLPDATDVWVIEPKTGRKLAKLECLDSRGFFSGV
H.influenzae NGDPFATLGMHETEQGIEIR-----
TLLPDANRMVVIERESGKEITELDCVDERGFFVGV
Agro.tume HSNPFQIIPLHETPEGFSAR-----CFIPGAEEVSVLTLD-
GNFVGELKQIDPDGFFEGR
Aquifex_a. ----------

Synechococcus EQNPFDILGPHPYEHEGQAG-WVIRAYLPEAQEAAVICPAL-
RREFAMHPVHHPHFFETW
Synechocystis
HHDPFEVLGCHPLGDHGKVNQWVIRAYLPTAEAVTVLLPTD-
RREVIMTTVHHPNFFECV
Butyrivibrio ----------
CHLAMYDIA QSSPQDLLGIVS-ESLNQDR---IVLFRPGAETVFVELRG----
KIQQAESHHSGIFSLP

Bstearothermophilus ----------
MIAANPTDLEVYLFHEGSLYKSYELFGAHV--
Bcaldolyticus ----------
MIAANPTDLEVYLFHEGRLYQSYELFGAHV--
Bsubtilis ----------
MAAASPTAHDVYLFHEGSLFKSYQLFGSHY--
mycobacterium LPFVD-
LIDYRLQVTYEGCEPHTVADAYRFLPTLGEVDLHLFAEGRHERLWEVLGAHPRS
Streptomyces LPLTG-VPDYRLLVTYDSDE-
IEVHDPYRFLPALGELDLHLIGEGRHEELWTALGSQP--
E. IPRRKNFFRYQLAVVWHGQQ-
NLIDDPYRFGPLIQEMDAWLLSEGTHLRPYETLGAHA--
H.influenzae IPNCRQFFAYQLQVFWGNEA-
QIIEDPYRFHPMIDDLEQWLLSEGSMLRPYEVLGAHF--
Agro.tume IDLSK-RQPVRYRACRDDAE-
WAVTDPYSFGPVLGPMDDYFVREGSICGYSTGWARIP--
Aquifex_a. ----------
MKKFSLISDYDVYLFKEGTHTRLYDKLGSHV--
Synechococcus VPEET-
LEIYQLRITEGERERIIYDPYAFRSPLLTDYDIHLFAEGNHHRIYEKLGAHP--
Synechocystis LELEE-
PKNYQLRITENGHERVIYDPYGFKTPKLTDFDLHVFGEGNHHRIYEKLGAHL--
Butyrivibrio ----------
MSQKVFISEDDEYLFGQGTHYDIYDKLGAHP--

CHLAMYDIA VMKGISPQDYRVYHQN-G---
LLAHDPYAFPLLWGEIDSFLFHEGTHQRIYERMGAIP--
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Bstearothermophilus -INEGG-
KVGTRFCVWAPHAREVRLVGSFNDWDGTDFRLEKVND-EGVWTIVVPENLEGH
Bcaldolyticus -IRGGG-
AVGTRFCVWAPHAREVRLVGSFNDWNGTNSPLTKVND-EGVWTIVVPENLEGH
Bsubtilis -RELNG-
KSGYEFCVWAPHASEVRVAGDFNSWSGEEHVMHRVND-NGIWTLFIPGIGEKE
mycobacterium
FTTADGVVSGVSFAVWAPNAKGVSLIGEFNGWNGHEAPMRVLGP-
SGVWELFWPDFPCDG
Streptomyces -MEHQG-
VAGTRFTVWAPNALGVRVTGDFSYWDAVAYPMRSLGA-SGVWELFLPGVAEGA
E. -DTMDG-
VTGTRFSVWAPNARRVSVVGQFNYWDGRRHPMRLRKE-SGIWELFIPGAHNGQ
H.influenzae -MECDG-
VSGVNFRLWAPNARRVSIVGDFNYWDGRRHPMRFHSK-SGVWELFLPKASLGQ
Agro.tume -LKLEG-
VEGFHFAVWAPNGRRVSVVGDFNNWDGRRHVMRFRKD-TGIWEIFAPDVYA-C
Aquifex_a. -IELNG-
KRYTFFAVWAPHADYVSLIGDFNEWDKGSTPMVKREDGSGIWEVLLEGDLTGS
Synechococcus -CELEN-
VAGVNFAVWAPSARNVSILGDFNSWDGRKHQMAR-RS-NGIWELFIPELTVGA
Synechocystis -MTVDG-
VKGVYFAVWAPNARNVSILGDFNNWDGRLHQMRK-RN-NMVWELFIPELGVGT
Butyrivibrio -SEEKG-
KKGFFFAVWAPNAADVHVVGDFNGWDENAHQMKRSKT-GNIWTLFIPGVAIGA
CHLAMYDIA -CEIDG-
VPGVRFIVWAPHAQRVSVIGDFNGWHGLVNPLHKVSD-QGVWELFVPGLTAGA
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Bstearothermophilus LYKYEIVTPDGQVL-
FKADPYAFYSELRPHTASIAYDLKGYQWNDQSWKRKKRRKRIYDQ
Bcaldolyticus LYKYEIITPDGRVL-
LKADPYAFYSELRPHTASIVYDLKGYEWNDSPWQRKKRRKRIYDQ
Bsubtilis RYKYEIVTNNGEIR-
LKADPYAIYSEVRPNTASLTYDLEGYSWQDQKWQKKQKAKTLYEK
mycobacterium LYKFRVHGADGVVT-DRADPFAFGTEVPPQTASRVT-
SSDYTWGDDDWMAGRALRNPVNE
Streptomyces LYKYEITRPDGGRT-LRADPMARYAEVPPANASIVT-
ASRYEWQDAEWMARRGALAPHQA
E. LYKYEMIDANGNLR-LKSDPYAFEAQMRPETASLIC-
GLPEKVVQTEERKKANQFDA---
H.influenzae LYKFELIDCHGNLR-LKADPFAFSSQLRPDTASQVS-
ALPNVVEMTEARKKANQGNQ---
Agro.tume
AYKFEILGANGELLPLKADPYARRGELRPKNASVTAPELTQKWEDQAHREHWAQ
VDQRRQ
Aquifex_a. KYKYFIKNGNYEVD--KSDPFAFFCEQPPGNASVVW-
KLNYRWNDSEYMKKRKRVNSHDS
Synechococcus AYKYEIKNYDGHIYE-
KSDPYGFQQEVRPKTASIVADLDRYTWGDADWLERRRHQEPLRQ
Synechocystis SYKYEIKNWEGHIYE-
KTDPYGFYQEVRPKTASIVADLDGYQWHDEDWLEARRTSDPLSK
Butyrivibrio LYKFLITAQDGRKLY-
KADPYANYAELRPGNASRTTDLSGFKWSDSKWYESLKGKDMNRQ
CHLAMYDIA CYKWEMVTESGQVL-IKSDPYGKFFGPPPWSVSVVI-
DDSYEWTDSEWLEERIKKTEG--
: : :: . * ..*
Bstearothermophilus PMVIYELHFGSWKKK----------
DGRFYTYREMADELISYVLDH
Bcaldolyticus PMVIYELHFGSWKKKP----------
DGRFYTYREMADELIPYVLER
Bsubtilis PVFIYELHLGSWKKHS----------
DGRHYSYKELSQTLIPYIKKH

mycobacterium AMSTYEVHLGSWRP----------
GLSYRQLARELTDYIVDQ
Streptomyces PMSVYELHLASWRP----------
GLSYRQLAEQLPAYVKEL
E. PISIYEVHLGSWRRH----------TDNN----------
FWLSYRELADQLVPYAKWM
H.influenzae PISIYEVHLGSWRRN----------LENN----------
FWLDYDQIADELIPYVKEM
Agro.tume PISIYEVHAGSWQR----------SEDG----------
TFLSWDELEAQLIPYCTDM
Aquifex_a. PISIYEVHVGSWRRVP----------EEGN----------
RFLSYRELAEYLPYYVKEM
Synechococcus
PISVYEVHLGSWMHASSDAIATDAQGKPLPPVPVADLKPGARFLTYRELADRLIPY
VLDL
Synechocystis PVSVYELHLGSWLHTAYDEPVKTLHGEGVP-
VEVSEWNTGARFLTYYELVDKLIPYVKEL
Butyrivibrio PIAIYECHIGSWMKHP--------DGTEDG----------
FYTYRQFADRIVEYLKEM
CHLAMYDIA PMNIYEVHVGSWRWQE----------
GQPLNYKELADQLALYCKQM
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Bstearothermophilus
GFTHIELLPLVEHPLDRSWGYQGTGYYAVTSRYGTPHDFMYFVDRCHQAGIGVIM
DWVPG
Bcaldolyticus
GFTHIELLPLVEHPLDRSWGYQGTGYYSVTSRYGTPHDFMYFVDRCHQAGLGVII
DWVPG
Bsubtilis
GFTHIELLPVYEHPYDRSWGYQGTGYYSPTSRFGPPHDLMKFVDECHQQNIGVIL
DWVPG

mycobacterium
GFTHVELLPVAEHPFAGSWGYQVTSYYAPTSRFGTPDDFRALVDALHQAGIGVIVD
WVPA
Streptomyces
GFTHVELMPVAEHPFGGSWGYQVTGFYAPTSRMGTPDDFRFLVDALHRAGIGVIV
DWVPA
E.
GFTHLELLPINEHPFDGSWGYQPTGLYAPTRRFGTRDDFRYFIDAAHAAGLNVILD
WVPG
H.influenzae
GFTHIEFLPLSEFPFDGSWGYQPLGLYSPTSRFGSPEAFRRLVKRAHEAGINVILD
WVPG
Agro.tume
GFTHIEFLPITEHPYDPSWGYQTTGLYAPTARFGDPEGFARFVNGAHKVGIGVLLD
WVPA
Aquifex_a.
GFTHVEFLPVMEHPFYGSWGYQITGYFAPTSRYGTPQDFMYLIDKLHQEGIGVILD
WVPS
Synechococcus
GYSHIELLPIAEHPFDGSWGYQVTGYYAATSRYGSPEDFMYFVDRCHQNGIGVILD
WVPG
Synechocystis
GYTHIELLPIAEHPFDGSWGYQVTGYYAPTSRFGSPEDFMYFVDQCHLNGIGVIID
WVPG
Butyrivibrio
KYTHIELIGIAEHPFDGSWGYQVTGYYAPTARYGEPTDFMYLINQLHKHGIGVILD
WVPA
CHLAMYDIA
HYTHVELLPVTEHPLNESWGYQTTGYYAPTSRYGSFEDLQYFIDTMHQHGIGVIL
DWVPG

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Bstearothermophilus
HFCKDAHGLYMFDGAPTYEYANEKDRENYVWGTANFDLGKPEVRSFLISNALFW
LEYYHI
Bcaldolyticus
HFCKDAHGLYMFDGAPTYEYANEKDRENYVWGTANFDLGKPEVRSFLISNALFW
LEYYHV
Bsubtilis
HFCKDAHGLYMFDGEPLYEYKEERDRENWLWGTANFDLGKPEVHSFLISNALY
WAEFYHI
mycobacterium
HFPKDAWALGRFDGTPLYEHSDPKRGEQLDWGTYVFDFGRPEVRNFLVANALY
WLQEFHI
Streptomyces
HFPRDDWALAEFDGRPLYEHQDPRRAAHPDWGTLEFDYGRKEVRNFLVANAVY
WCQEFHV
E.
HFPTDDFALAEFDGTNLYEHSDPREGYHQDWNTLIYNYGRREVSNFLVGNALYW
IERFGI
H.influenzae
HFPSDTHGLVAFDGTALYEHEDPREGYHQDWNTLIYNYGRNEVKNFLSSNALYW
LERFGV
Agro.tume
HFPTDEHGLRWFDGTALYEHADPRQGFHPDWNTAIYNFGRIEVMSYLINNALYW
AEKFHL
Aquifex_a.
HFPTDAHGLAYFDGTHLYEYEDWRKRWHPDWNSFVFDYGKPEVRSFLLSSAHF
WLDKYHA
Synechococcus
HFPKDGHGLAFFDGTHLYEHADSRQGEHREWGTLVFNYGRHEVRNFLAANALF
WFDKYHI
Synechocystis
HFPKDGHGLAFFDGTHLYEHGDPRKGEHKEWGTLIFNYGRNEVRNFLVANALF
WFDKYHI

Butyrivibrio
HFCPDEFGLACFDGTCIYEDPDPRKGEHPDWGTKIFNLAKPEVKNFLIANALYWI
RKFHI
CHLAMYDIA
HFPIDSFAMSGFDGTPLYEYTRNPSPLHPHWHTYTFDYAKPEVCNFLLGSVLFWI
DKMHV
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Bstearothermophilus DGFRVDAVANMLYWPNNDRL------YE----
NPYAVEFLRKLNEAVFAYDPNALMIAED
Bcaldolyticus DGFRVDAVANMLYWPNNDRL------YE----
NPYAVEFLRQLNEAVFAYDPNVWMIAED
Bsubtilis DGFRVDAVANILYWPNQDER------HT----
NPYAVDFLKKLNQTMREAYPHVMMIAED
mycobacterium
DGLRVDAVASMLYLDYSRPEGGWTPNVHGGRENLEAVQFLQEMNATAHKVAPGI
VTIAEE
Streptomyces
DGLRADAVASMLYLDYSRDEGDWSPNAHGGREDLDAVALLQEMNATVYRRFPGV
VTIAEE
E.
DALRVDAVASMIYRDYSRKEGEWIPNEFGGRENLEAIEFLRNTNRILGEQVSGAVT
MAEE
H.influenzae
DGIRVDAVASMIYRDYSRAEGEWIPNQYGGRENLEAIEFLKHTNWKIHSEMAGAI
SIAEE
Agro.tume
DGLRVDAVASMLYLDYSRKEGEWIPNEYGGRENLESVRFLQKMNSLVYGTHPGV
MTIAEE
Aquifex_a. DGLRVDAVASMLYLDYSRKE--
WVPNIYGGKENLEAIEFLRKFNESVYRNFPDVQTIAEE
Synechococcus
DGIRVDAVASMLYLDYNRKEGEWIPNEYGGRENIEAADFLRQVNHLIFSYFPGALS
IAEE

Synechocystis
DGMRVDAVASMLYLDYCREEGEWVANEYGGRENLEAADFLRQVNSVVYSYFPGI
LSIAEE
Butyrivibrio
DGLRVDAVASMLYLDYGKKDGQWVPNKYGDNKNLDAIEFFKHFNSVVRGTYPNI
LTIAEE
CHLAMYDIA
DGIRVDAVSSMLYLDYGRYAGEWVPNRYGGRENLDAIRFLQQFNTVIHEKYPGVL
TFAEE
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Bstearothermophilus
STDWPKVTAPTYEGGLGFNYKWNMGWMNDMLKYMETPPYERRHVHNQVTFSL
LYAYSENF
Bcaldolyticus
STDWPRVTAPTYDGGLGFNYKWNMGWMNDMLKYMETPPHERKYAHNQVSFSL
LYAYSENF
Bsubtilis
STEWPQVTGAVEEGGLGFHYKWNMGWMNDVLKYMETPPEERRHCHQLISFSLL
YAFSEHF
mycobacterium
STPWSGVTRPTNIGGLGFSMKWNMGWMHDTLDYVSRDPVYRSYHHHEMTFSML
YAFSENY
Streptomyces
STAWDGVTRPTDSGGLGFGLKWNMGWMHDTLRYVSKEPVHRKYHHHDMTFGM
VYAFSENF
E.
STDFPGVSRPQDMGGLGFWYKWNLGWMHDTLDYMKLDPVYRQYHHDKLTFGI
LYNYTENF
H.influenzae
STSFAGVTHPSENGGLGFNFKWNMGWMNDTLAYMKLDPIYRQYHHNKMTFGM
VYQYSENF

Agro.tume
STSWPKVSQPVHEGGLGFGFKWNMGFMHDTLSYFSREPVHRKFHHQELTFGLL
YAFTENF
Aquifex_a.
STAWPMVSRPTYVGGLGFGMKWNMGWMNDTLFYFSKDPIYRKYHHEVLTFSIW
YAFSENF
Synechococcus
STSWPMVSWPTYVGGLGFNLKWNMGWMHDMLDYFSMDPWFRQFHQNNVTFSI
WYAFSENF
Synechocystis
STSWPMVSWPTYVGGLGFNLKWNMGWMHDMLDYFSMDPWFRQFHQNSITFSM
WYNHSENY
Butyrivibrio
STAWPKVTAPPEEDGLGFAFKWNMGWMHDFCEYMKLDPYFRQGAHYMMTFAM
SYNDSENY
CHLAMYDIA
STTFPKITVSVEEGGLGFDYKWNMGWMHDTLHYFEKDFPYRPYHQSDLTFPQW
YAFSERF
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Bstearothermophilus
ILPFSHDEVVHGKKSLLNKMPGSYEEKFAQLRLLYGYMMAHPGKKLLFMGNEFA
QFDEWK
Bcaldolyticus
ILPFSHDEVVHGKKSLLNKMPGSYEEKFAQLRLLYGYMMAHPGKKLLFMGSEFA
QFDEWK
Bsubtilis
VLPFSHDEVVYGKKSLLNKMPGDYWQKFAQYRLLLGYMTVHPGKKLIFMGSEFA
QFDEWK
mycobacterium
VLPLSHDEVVHGKGTLWGRMPGNNHVKAAGLRSLLAYQWAHPGKQLLFMGQEF
GQRAEWS
Streptomyces
VLPISHDEVVHGKRSLVSKMPGDWWQQRATHRAYLGFMWAHPGKQLLFMGQEF
AQGSEWS

E.
VLPLSHDEVVHGKKSILDRMPGDAWQKFANLRAYYGWMWAFPGKKLLFMGNEF
AQGREWN
H.influenzae
VLPLSHDEVVHGKYSLLGKMPGDTWQKFANLRAYYGYMWGYPGKKLLFMGNEF
AQGREWN
Agro.tume
VLPLSHDEVVHGKGSLIAKMSGDDWQKFANLRSYYGFMWGYPGKKLLFMGQEF
AQWSEWS
Aquifex_a.
VLPLSHDEVVHGKGSLIGKMPGDYWQKFANLRALFGYMWAHPGKKLLFMGGEF
GQFKEWD
Synechococcus
MLALSHDEVVHGKSNLIGKMPGDEWQKFANLRCLLGYMFTHPGKKTLFMGMEF
GQWAEWN
Synechocystis
MLALSHDEVVHGKSNMLGKMPGDEWQKYANVRALFTYMFTHPGKKTMFMSME
FGQWSEWN
Butyrivibrio
ILPLSHDEVVHLKCSMVEKMPGYKVDKYANLRVGYTYMFGHSGKKLLFMGQDF
GQEREWS
CHLAMYDIA
LLPFSHDEVVHGKRSLIGKMPGDAWRQFAQLRLLLGYQICQPGKKLLFMGGEFG
QGREWS
:*.:******:*.:.:*.* :* * : .**:.:**.:*.* **.

Bstearothermophilus FEDELDWVLFDF-----
ELHRKMNDYMKELIACYKRYKPFYELDHDPQGFEWIDVHNAEQ
Bcaldolyticus FAEELDWVLFDF-----
ELHRKMDEYVKQLIACYKRYKPFYELDHDPRGFEWIDVHNAEQ
Bsubtilis DTEQLDWFLDSF-----
PMHQKASVFTQDLLRFYQKSKILYEHDHRAQSFEWIDVHNDEQ
mycobacterium EQRGLDWFQLDE----
NGFSNGIQRLVRDINDIYRCHPALWSLDTTPEGYSWIDANDSAN

Streptomyces
ETYGPDWWVLDSSYPAAGDHLGVRSLVRDLNRTYTASPALWERDSVPEGFAWVE
ADAADD
E. HDASLDWHLLEG---
GDNWHHGVQRLVRDLNLTYRHHKAMHELDFDPYGFEWLVVDDKER
H.influenzae YEESLDWFLLDENI-
GGGWHKGVLKLVKDLNQIYQKNRPLFELDNSPEGFDWLVVDDAAN
Agro.tume EKGSLDWNLRQY-----
PMHEGMRRLVRDLNLTYRSKAALHARDCEPDGFRWLVVDDHEN
Aquifex_a. HETSLDWHLLEY-----
PSHRGIQRLVKDLNEVYRREKALHETDFSPEGFEWVDFHDWEK
Synechococcus VWGDLEWHLLQY-----
EPHQGLKQFVKDLNHLYRNAPALYSEDCNQAGFEWIDCSDNRH
Synechocystis VWGDLEWHLLNF-----
PPHQQLKQFFTELNHLYKNEPALYSNDFDESGFQWIDCSDNRH
Butyrivibrio EKRELDWFLLEN-----
DLNRGMKDYVGKLLEIYRKYPALYEVDNDWGGFEWINADDKER
CHLAMYDIA PGRELDWELLDI-----
SYHQGVHLCSQELNALYVQSPQLWQADHLPSSFRWVDFSDVRN
:* . .: * : * .: *:

Bstearothermophilus SIFSFIRRGKKED-DVLVIVCNFTNQAYDDYKVGVP-
LLVPYREVLNSDAVTFGGSGHVN
Bcaldolyticus SIFSFIRRGKKEG-DVLVIVCNFTNQAYDDYKVSVP-
LLAPYREVLNSDAAEFGGSGHVN
Bsubtilis SIFSFIRYGQKHG-EALVIICNFTPVVYHQYDVGVP-
FFTQYIEVLNSDSETYGGSGQIN
mycobacterium NVLSFMRYGSDG--SVLACVFNFAGAEHRDYRLGLP-
RAGRWREVLNTDATIYHGSGIGN
Streptomyces NVFAFLRFARDG--
SPLLCVSNFSPVVRHGYRIGVPQEVGQWREVLNTDLEPYGGSGVHH
E. SVLIFVRRDKEG--NEIIVASNFTPVPRHDYRFGIN-
QPGKWREILNTDSMHYHGSNAGN

H.influenzae SVLAFERRSSNG--ERIIVVSNFTPVPRHNYRIGVN-
VAGKYEEILNTDSMYYEGSNVGN
Agro.tume SVFAWLRTAPGE--KPVAVICNLTPVYRENYYVPLG-
VAGRWREILNTDAEIYGGSGKGN
Aquifex_a. SVISFLRKDKSGK-EIILVVCNFTPVPRYDYRVGVP-
KGGYWREIMNTDAKEYWGSGMGN
Synechococcus SIVSFIRRAHESD-RFLVVVCNFTPQPHAHYRIGVP-
VAGFYREIFNSDARSYGGSNMGN
Synechocystis SVVSFIRRAKNSA-EFVVTICNFTPQPHSHYRVGVP-
VPGFYTELFNSDARQYGGSNMGN
Butyrivibrio STYSFYRRASNGK-DNILFVLNMTPMERKGFKVGVP-
FDGTYTKILDSAKECYGGSGSSV
CHLAMYDIA GVVAYLRFADADAKKALLCVHHFGVGYFPHYLLPIL-
PLESCDLLMNTDDTRFGGSGKGF
. . : * : :: : . : :::: : **.

Bstearothermophilus GKR-LSAFNEPFHGK----------P--
YHVRMTIPPFGISILRPVQKRGERKRNEK
Bcaldolyticus GKR-LPAFSEPFHGK----------P--
YHVRMTIPPFGISILRPVQKRGERKQNEE
Bsubtilis KKP-LSAKKGALHHK----------P--
CYITMTIPPYGISILRAVKKRGEIKR---
mycobacterium LGG-VDATDDPWHGR----------P--
ASAVLVLPPTSALWLTPA----------
Streptomyces ARA-LRPEPVPAQGR----------A--VSLRMTLPPMATVWLRP----
----
E. GGT-VHSDEIASHGR----------Q--HSLSLTLPPLATIWLVREAE-------
--
H.influenzae FGC-VASEQIESHGR----------E--NSISVSIPPLATVYLRLKTK--
----
Agro.tume GG----RVQAVDAGG----------E--IGAMLVLPPLATIMLEPEN-----
-----
Aquifex_a. LGG-KEADKIPWHGR----------K--FSLSLTLPPLSVIYLKHEG---
----

Synechococcus LGG-KWTDEWSCHNR----------P--
YSLDLCLPPLTTLVLELASGPES----LS
Synechocystis LGG-KWTEEWSFHEQ----------P--
YSLDLCLPPLSVLVLKLSQNAEENTVPAE
Butyrivibrio PDK-IKAVKGLCDYK----------D--
YSIEFDLPPYGAEVFVFQTKKTKN-----
CHLAMYDIA
REPEILTPEIARQEREAAGLIEADDESGPDCWGLDIELPPSATLIFSVTLQ---------
. :** :

Bstearothermophilus EMHRHVIGRRARKSASLADDKHR----------
Bcaldolyticus EVHRHVIGRRARKPASLADEKHRETSRAVWGEVPDH
Bsubtilis ----------
mycobacterium ----------
Streptomyces ----------
E. ----------
H.influenzae ----------
Agro.tume ----------
Aquifex_a. ----------
Synechococcus EAANSPL----------
Synechocystis EASNIA----------
Butyrivibrio ----------
CHLAMYDIA ----------

Alignement of BE and isoamylases.

Bstearothermophilus ----------
Bcaldolyticus ----------
Bsubtilis ----------
mycobacterium ----------MSRSEKLTGEH-LAPEPA----
EMARLVAGT
Streptomyces
MSAARQPSPTVRDKAAPEPAAPAAPKGARAPRARRAAPPHGVRPAPALAAEERAR
LLEGR
E. ----------MSDRIDRDVINALIAGH
H.influenzae ----------MTTAVTQAIIDGFFDAS
Agro.tume ----------MKKPLNSAEEKKTGDITKAEIEAIKSGL
Aquifex_a. ----------
Synechococcus ----------TGTTPLPSSSLSVEQVNRIASNQ
Synechocystis ----------MTYTINADQVHQIVHNL
Butyrivibrio ----------
CHLAMYDIA ----------MDPFFLNTQHVELLVSGK

Bstearothermophilus ----------
Bcaldolyticus ----------
Bsubtilis ----------
mycobacterium HHNPHGILGAHEYDDHTVIR-----AFRPHAVEVVALVGK---
DRFSLQHLD-SGLFAVA
Streptomyces HHDPHAVLGARTQRGGVAFR-----VLRPYAKAVTVVAKG---
LRTELVDEG-DGLFSGL
E. FADPFSVLGMHKTTAGLEVR-----
ALLPDATDVWVIEPKTGRKLAKLECLDSRGFFSGV
H.influenzae NGDPFATLGMHETEQGIEIR-----
TLLPDANRMVVIERESGKEITELDCVDERGFFVGV
Agro.tume HSNPFQIIPLHETPEGFSAR-----CFIPGAEEVSVLTLD-
GNFVGELKQIDPDGFFEGR

Aquifex_a. ----------
Synechococcus EQNPFDILGPHPYEHEGQAG-WVIRAYLPEAQEAAVICPAL-
RREFAMHPVHHPHFFETW
Synechocystis
HHDPFEVLGCHPLGDHGKVNQWVIRAYLPTAEAVTVLLPTD-
RREVIMTTVHHPNFFECV
Butyrivibrio ----------
CHLAMYDIA QSSPQDLLGIVS-ESLNQDR---IVLFRPGAETVFVELRG----
KIQQAESHHSGIFSLP

Bstearothermophilus ----------
MIAANPTDLEVYLFHEGSLYKSYELFGAHV--
Bcaldolyticus ----------
MIAANPTDLEVYLFHEGRLYQSYELFGAHV--
Bsubtilis ----------
MAAASPTAHDVYLFHEGSLFKSYQLFGSHY--
mycobacterium LPFVD-
LIDYRLQVTYEGCEPHTVADAYRFLPTLGEVDLHLFAEGRHERLWEVLGAHPRS
Streptomyces LPLTG-VPDYRLLVTYDSDE-
IEVHDPYRFLPALGELDLHLIGEGRHEELWTALGSQP--
E. IPRRKNFFRYQLAVVWHGQQ-
NLIDDPYRFGPLIQEMDAWLLSEGTHLRPYETLGAHA--
H.influenzae IPNCRQFFAYQLQVFWGNEA-
QIIEDPYRFHPMIDDLEQWLLSEGSMLRPYEVLGAHF--
Agro.tume IDLSK-RQPVRYRACRDDAE-
WAVTDPYSFGPVLGPMDDYFVREGSICGYSTGWARIP--
Aquifex_a. ----------
MKKFSLISDYDVYLFKEGTHTRLYDKLGSHV--
Synechococcus VPEET-
LEIYQLRITEGERERIIYDPYAFRSPLLTDYDIHLFAEGNHHRIYEKLGAHP--
Synechocystis LELEE-
PKNYQLRITENGHERVIYDPYGFKTPKLTDFDLHVFGEGNHHRIYEKLGAHL--

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Butyrivibrio            ----------
MSQKVFISEDDEYLFGQGTHYDIYDKLGAHP--
CHLAMYDIA               VMKGISPQDYRVYHQN-G---
LLAHDPYAFPLLWGEIDSFLFHEGTHQRIYERMGAIP--
                                  : .. :*       .

Bstearothermophilus     -INEGG-
KVGTRFCVWAPHAREVRLVGSFNDWDGTDFRLEKVND-EGVWTIVVPENLEGH
Bcaldolyticus           -IRGGG-
AVGTRFCVWAPHAREVRLVGSFNDWNGTNSPLTKVND-EGVWTIVVPENLEGH
Bsubtilis               -RELNG-
KSGYEFCVWAPHASEVRVAGDFNSWSGEEHVMHRVND-NGIWTLFIPGIGEKE
mycobacterium
FTTADGVVSGVSFAVWAPNAKGVSLIGEFNGWNGHEAPMRVLGP-
SGVWELFWPDFPCDG
Streptomyces            -MEHQG-
VAGTRFTVWAPNALGVRVTGDFSYWDAVAYPMRSLGA-SGVWELFLPGVAEGA
E.                      -DTMDG-
VTGTRFSVWAPNARRVSVVGQFNYWDGRRHPMRLRKE-SGIWELFIPGAHNGQ
H.influenzae            -MECDG-
VSGVNFRLWAPNARRVSIVGDFNYWDGRRHPMRFHSK-SGVWELFLPKASLGQ
Agro.tume               -LKLEG-
VEGFHFAVWAPNGRRVSVVGDFNNWDGRRHVMRFRKD-TGIWEIFAPDVYA-C
Aquifex_a.              -IELNG-
KRYTFFAVWAPHADYVSLIGDFNEWDKGSTPMVKREDGSGIWEVLLEGDLTGS
Synechococcus           -CELEN-
VAGVNFAVWAPSARNVSILGDFNSWDGRKHQMAR-RS-NGIWELFIPELTVGA
Synechocystis           -MTVDG-
VKGVYFAVWAPNARNVSILGDFNNWDGRLHQMRK-RN-NMVWELFIPELGVGT
Butyrivibrio            -SEEKG-
KKGFFFAVWAPNAADVHVVGDFNGWDENAHQMKRSKT-GNIWTLFIPGVAIGA
CHLAMYDIA               -CEIDG-
VPGVRFIVWAPHAQRVSVIGDFNGWHGLVNPLHKVSD-QGVWELFVPGLTAGA
                  .     * :*** .  * : *.*. *     :      :* :.
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Bstearothermophilus LYKYEIVTPDGQVL-
FKADPYAFYSELRPHTASIAYDLKGYQWNDQSWKRKKRRKRIYDQ
Bcaldolyticus LYKYEIITPDGRVL-
LKADPYAFYSELRPHTASIVYDLKGYEWNDSPWQRKKRRKRIYDQ
Bsubtilis RYKYEIVTNNGEIR-
LKADPYAIYSEVRPNTASLTYDLEGYSWQDQKWQKKQKAKTLYEK
mycobacterium LYKFRVHGADGVVT-DRADPFAFGTEVPPQTASRVT-
SSDYTWGDDDWMAGRALRNPVNE
Streptomyces LYKYEITRPDGGRT-LRADPMARYAEVPPANASIVT-
ASRYEWQDAEWMARRGALAPHQA
E. LYKYEMIDANGNLR-LKSDPYAFEAQMRPETASLIC-
GLPEKVVQTEERKKANQFDA---
H.influenzae LYKFELIDCHGNLR-LKADPFAFSSQLRPDTASQVS-
ALPNVVEMTEARKKANQGNQ---
Agro.tume
AYKFEILGANGELLPLKADPYARRGELRPKNASVTAPELTQKWEDQAHREHWAQ
VDQRRQ
Aquifex_a. KYKYFIKNGNYEVD--KSDPFAFFCEQPPGNASVVW-
KLNYRWNDSEYMKKRKRVNSHDS
Synechococcus AYKYEIKNYDGHIYE-
KSDPYGFQQEVRPKTASIVADLDRYTWGDADWLERRRHQEPLRQ
Synechocystis SYKYEIKNWEGHIYE-
KTDPYGFYQEVRPKTASIVADLDGYQWHDEDWLEARRTSDPLSK
Butyrivibrio LYKFLITAQDGRKLY-
KADPYANYAELRPGNASRTTDLSGFKWSDSKWYESLKGKDMNRQ
CHLAMYDIA CYKWEMVTESGQVL-IKSDPYGKFFGPPPWSVSVVI-
DDSYEWTDSEWLEERIKKTEG--
:: :: . * ..*
Bstearothermophilus PMVIYELHFGSWKKK----------
DGRFYTYREMADELISYVLDH
Bcaldolyticus PMVIYELHFGSWKKKP----------
DGRFYTYREMADELIPYVLER

Bsubtilis PVFIYELHLGSWKKHS----------
DGRHYSYKELSQTLIPYIKKH
mycobacterium AMSTYEVHLGSWRP----------
GLSYRQLARELTDYIVDQ
Streptomyces PMSVYELHLASWRP----------
GLSYRQLAEQLPAYVKEL
E. PISIYEVHLGSWRRH----------TDNN----------
FWLSYRELADQLVPYAKWM
H.influenzae PISIYEVHLGSWRRN----------LENN----------
FWLDYDQIADELIPYVKEM
Agro.tume PISIYEVHAGSWQR----------SEDG----------
TFLSWDELEAQLIPYCTDM
Aquifex_a. PISIYEVHVGSWRRVP----------EEGN----------
RFLSYRELAEYLPYYVKEM
Synechococcus
PISVYEVHLGSWMHASSDAIATDAQGKPLPPVPVADLKPGARFLTYRELADRLIPY
VLDL
Synechocystis PVSVYELHLGSWLHTAYDEPVKTLHGEGVP-
VEVSEWNTGARFLTYYELVDKLIPYVKEL
Butyrivibrio PIAIYECHIGSWMKHP--------DGTEDG----------
FYTYRQFADRIVEYLKEM
CHLAMYDIA PMNIYEVHVGSWRWQE----------
GQPLNYKELADQLALYCKQM
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Bstearothermophilus
GFTHIELLPLVEHPLDRSWGYQGTGYYAVTSRYGTPHDFMYFVDRCHQAGIGVIM
DWVPG
Bcaldolyticus
GFTHIELLPLVEHPLDRSWGYQGTGYYSVTSRYGTPHDFMYFVDRCHQAGLGVII
DWVPG
Bsubtilis
GFTHIELLPVYEHPYDRSWGYQGTGYYSPTSRFGPPHDLMKFVDECHQQNIGVIL
DWVPG

mycobacterium
GFTHVELLPVAEHPFAGSWGYQVTSYYAPTSRFGTPDDFRALVDALHQAGIGVIVD
WVPA
Streptomyces
GFTHVELMPVAEHPFGGSWGYQVTGFYAPTSRMGTPDDFRFLVDALHRAGIGVIV
DWVPA
E.
GFTHLELLPINEHPFDGSWGYQPTGLYAPTRRFGTRDDFRYFIDAAHAAGLNVILD
WVPG
H.influenzae
GFTHIEFLPLSEFPFDGSWGYQPLGLYSPTSRFGSPEAFRRLVKRAHEAGINVILD
WVPG
Agro.tume
GFTHIEFLPITEHPYDPSWGYQTTGLYAPTARFGDPEGFARFVNGAHKVGIGVLLD
WVPA
Aquifex_a.
GFTHVEFLPVMEHPFYGSWGYQITGYFAPTSRYGTPQDFMYLIDKLHQEGIGVILD
WVPS
Synechococcus
GYSHIELLPIAEHPFDGSWGYQVTGYYAATSRYGSPEDFMYFVDRCHQNGIGVILD
WVPG
Synechocystis
GYTHIELLPIAEHPFDGSWGYQVTGYYAPTSRFGSPEDFMYFVDQCHLNGIGVIID
WVPG
Butyrivibrio
KYTHIELIGIAEHPFDGSWGYQVTGYYAPTARYGEPTDFMYLINQLHKHGIGVILD
WVPA
CHLAMYDIA
HYTHVELLPVTEHPLNESWGYQTTGYYAPTSRYGSFEDLQYFIDTMHQHGIGVIL
DWVPG

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Bstearothermophilus
HFCKDAHGLYMFDGAPTYEYANEKDRENYVWGTANFDLGKPEVRSFLISNALFW
LEYYHI
Bcaldolyticus
HFCKDAHGLYMFDGAPTYEYANEKDRENYVWGTANFDLGKPEVRSFLISNALFW
LEYYHV
Bsubtilis
HFCKDAHGLYMFDGEPLYEYKEERDRENWLWGTANFDLGKPEVHSFLISNALY
WAEFYHI
mycobacterium
HFPKDAWALGRFDGTPLYEHSDPKRGEQLDWGTYVFDFGRPEVRNFLVANALY
WLQEFHI
Streptomyces
HFPRDDWALAEFDGRPLYEHQDPRRAAHPDWGTLEFDYGRKEVRNFLVANAVY
WCQEFHV
E.
HFPTDDFALAEFDGTNLYEHSDPREGYHQDWNTLIYNYGRREVSNFLVGNALYW
IERFGI
H.influenzae
HFPSDTHGLVAFDGTALYEHEDPREGYHQDWNTLIYNYGRNEVKNFLSSNALYW
LERFGV
Agro.tume
HFPTDEHGLRWFDGTALYEHADPRQGFHPDWNTAIYNFGRIEVMSYLINNALYW
AEKFHL
Aquifex_a.
HFPTDAHGLAYFDGTHLYEYEDWRKRWHPDWNSFVFDYGKPEVRSFLLSSAHF
WLDKYHA
Synechococcus
HFPKDGHGLAFFDGTHLYEHADSRQGEHREWGTLVFNYGRHEVRNFLAANALF
WFDKYHI
Synechocystis
HFPKDGHGLAFFDGTHLYEHGDPRKGEHKEWGTLIFNYGRNEVRNFLVANALF
WFDKYHI

Butyrivibrio
HFCPDEFGLACFDGTCIYEDPDPRKGEHPDWGTKIFNLAKPEVKNFLIANALYWI
RKFHI
CHLAMYDIA
HFPIDSFAMSGFDGTPLYEYTRNPSPLHPHWHTYTFDYAKPEVCNFLLGSVLFWI
DKMHV
** * .: *** ** : * : :: .: ** .:* .. :*

Bstearothermophilus DGFRVDAVANMLYWPNNDRL------YE----
NPYAVEFLRKLNEAVFAYDPNALMIAED
Bcaldolyticus DGFRVDAVANMLYWPNNDRL------YE----
NPYAVEFLRQLNEAVFAYDPNVWMIAED
Bsubtilis DGFRVDAVANILYWPNQDER------HT----
NPYAVDFLKKLNQTMREAYPHVMMIAED
mycobacterium
DGLRVDAVASMLYLDYSRPEGGWTPNVHGGRENLEAVQFLQEMNATAHKVAPGI
VTIAEE
Streptomyces
DGLRADAVASMLYLDYSRDEGDWSPNAHGGREDLDAVALLQEMNATVYRRFPGV
VTIAEE
E.
DALRVDAVASMIYRDYSRKEGEWIPNEFGGRENLEAIEFLRNTNRILGEQVSGAVT
MAEE
H.influenzae
DGIRVDAVASMIYRDYSRAEGEWIPNQYGGRENLEAIEFLKHTNWKIHSEMAGAI
SIAEE
Agro.tume
DGLRVDAVASMLYLDYSRKEGEWIPNEYGGRENLESVRFLQKMNSLVYGTHPGV
MTIAEE
Aquifex_a. DGLRVDAVASMLYLDYSRKE--
WVPNIYGGKENLEAIEFLRKFNESVYRNFPDVQTIAEE
Synechococcus
DGIRVDAVASMLYLDYNRKEGEWIPNEYGGRENIEAADFLRQVNHLIFSYFPGALS
IAEE

Synechocystis
DGMRVDAVASMLYLDYCREEGEWVANEYGGRENLEAADFLRQVNSVVYSYFPGI
LSIAEE
Butyrivibrio
DGLRVDAVASMLYLDYGKKDGQWVPNKYGDNKNLDAIEFFKHFNSVVRGTYPNI
LTIAEE
CHLAMYDIA
DGIRVDAVSSMLYLDYGRYAGEWVPNRYGGRENLDAIRFLQQFNTVIHEKYPGVL
TFAEE
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Bstearothermophilus
STDWPKVTAPTYEGGLGFNYKWNMGWMNDMLKYMETPPYERRHVHNQVTFSL
LYAYSENF
Bcaldolyticus
STDWPRVTAPTYDGGLGFNYKWNMGWMNDMLKYMETPPHERKYAHNQVSFSL
LYAYSENF
Bsubtilis
STEWPQVTGAVEEGGLGFHYKWNMGWMNDVLKYMETPPEERRHCHQLISFSLL
YAFSEHF
mycobacterium
STPWSGVTRPTNIGGLGFSMKWNMGWMHDTLDYVSRDPVYRSYHHHEMTFSML
YAFSENY
Streptomyces
STAWDGVTRPTDSGGLGFGLKWNMGWMHDTLRYVSKEPVHRKYHHHDMTFGM
VYAFSENF
E.
STDFPGVSRPQDMGGLGFWYKWNLGWMHDTLDYMKLDPVYRQYHHDKLTFGI
LYNYTENF
H.influenzae
STSFAGVTHPSENGGLGFNFKWNMGWMNDTLAYMKLDPIYRQYHHNKMTFGM
VYQYSENF

Agro.tume
STSWPKVSQPVHEGGLGFGFKWNMGFMHDTLSYFSREPVHRKFHHQELTFGLL
YAFTENF
Aquifex_a.
STAWPMVSRPTYVGGLGFGMKWNMGWMNDTLFYFSKDPIYRKYHHEVLTFSIW
YAFSENF
Synechococcus
STSWPMVSWPTYVGGLGFNLKWNMGWMHDMLDYFSMDPWFRQFHQNNVTFSI
WYAFSENF
Synechocystis
STSWPMVSWPTYVGGLGFNLKWNMGWMHDMLDYFSMDPWFRQFHQNSITFSM
WYNHSENY
Butyrivibrio
STAWPKVTAPPEEDGLGFAFKWNMGWMHDFCEYMKLDPYFRQGAHYMMTFAM
SYNDSENY
CHLAMYDIA
STTFPKITVSVEEGGLGFDYKWNMGWMHDTLHYFEKDFPYRPYHQSDLTFPQW
YAFSERF
** : :: . .**** ***:*:*:* *.. * : ::* * :*.: .
Bstearothermophilus
ILPFSHDEVVHGKKSLLNKMPGSYEEKFAQLRLLYGYMMAHPGKKLLFMGNEFA
QFDEWK
Bcaldolyticus
ILPFSHDEVVHGKKSLLNKMPGSYEEKFAQLRLLYGYMMAHPGKKLLFMGSEFA
QFDEWK
Bsubtilis
VLPFSHDEVVYGKKSLLNKMPGDYWQKFAQYRLLLGYMTVHPGKKLIFMGSEFA
QFDEWK
mycobacterium
VLPLSHDEVVHGKGTLWGRMPGNNHVKAAGLRSLLAYQWAHPGKQLLFMGQEF
GQRAEWS
Streptomyces
VLPISHDEVVHGKRSLVSKMPGDWWQQRATHRAYLGFMWAHPGKQLLFMGQEF
AQGSEWS

E.
VLPLSHDEVVHGKKSILDRMPGDAWQKFANLRAYYGWMWAFPGKKLLFMGNEF
AQGREWN
H.influenzae
VLPLSHDEVVHGKYSLLGKMPGDTWQKFANLRAYYGYMWGYPGKKLLFMGNEF
AQGREWN
Agro.tume
VLPLSHDEVVHGKGSLIAKMSGDDWQKFANLRSYYGFMWGYPGKKLLFMGQEF
AQWSEWS
Aquifex_a.
VLPLSHDEVVHGKGSLIGKMPGDYWQKFANLRALFGYMWAHPGKKLLFMGGEF
GQFKEWD
Synechococcus
MLALSHDEVVHGKSNLIGKMPGDEWQKFANLRCLLGYMFTHPGKKTLFMGMEF
GQWAEWN
Synechocystis
MLALSHDEVVHGKSNMLGKMPGDEWQKYANVRALFTYMFTHPGKKTMFMSME
FGQWSEWN
Butyrivibrio
ILPLSHDEVVHLKCSMVEKMPGYKVDKYANLRVGYTYMFGHSGKKLLFMGQDF
GQEREWS
CHLAMYDIA
LLPFSHDEVVHGKRSLIGKMPGDAWRQFAQLRLLLGYQICQPGKKLLFMGGEFG
QGREWS
:*.:******: * .: :*.* : * * : .**: :**. :*.* **.

Bstearothermophilus FEDELDWVLFDF-----
ELHRKMNDYMKELIACYKRYKPFYELDHDPQGFEWIDVHNAEQ
Bcaldolyticus FAEELDWVLFDF-----
ELHRKMDEYVKQLIACYKRYKPFYELDHDPRGFEWIDVHNAEQ
Bsubtilis DTEQLDWFLDSF-----
PMHQKASVFTQDLLRFYQKSKILYEHDHRAQSFEWIDVHNDEQ
mycobacterium EQRGLDWFQLDE----
NGFSNGIQRLVRDINDIYRCHPALWSLDTTPEGYSWIDANDSAN

Streptomyces
ETYGPDWWVLDSSYPAAGDHLGVRSLVRDLNRTYTASPALWERDSVPEGFAWVE
ADAADD
E. HDASLDWHLLEG---
GDNWHHGVQRLVRDLNLTYRHHKAMHELDFDPYGFEWLVVDDKER
H.influenzae YEESLDWFLLDENI-
GGGWHKGVLKLVKDLNQIYQKNRPLFELDNSPEGFDWLVVDDAAN
Agro.tume EKGSLDWNLRQY-----
PMHEGMRRLVRDLNLTYRSKAALHARDCEPDGFRWLVVDDHEN
Aquifex_a. HETSLDWHLLEY-----
PSHRGIQRLVKDLNEVYRREKALHETDFSPEGFEWVDFHDWEK
Synechococcus VWGDLEWHLLQY-----
EPHQGLKQFVKDLNHLYRNAPALYSEDCNQAGFEWIDCSDNRH
Synechocystis VWGDLEWHLLNF-----
PPHQQLKQFFTELNHLYKNEPALYSNDFDESGFQWIDCSDNRH
Butyrivibrio EKRELDWFLLEN-----
DLNRGMKDYVGKLLEIYRKYPALYEVDNDWGGFEWINADDKER
CHLAMYDIA PGRELDWELLDI-----
SYHQGVHLCSQELNALYVQSPQLWQADHLPSSFRWVDFSDVRN
:* . .: * : * .: *:

Bstearothermophilus SIFSFIRRGKKED-DVLVIVCNFTNQAYDDYKVGVP-
LLVPYREVLNSDAVTFGGSGHVN
Bcaldolyticus SIFSFIRRGKKEG-DVLVIVCNFTNQAYDDYKVSVP-
LLAPYREVLNSDAAEFGGSGHVN
Bsubtilis SIFSFIRYGQKHG-EALVIICNFTPVVYHQYDVGVP-
FFTQYIEVLNSDSETYGGSGQIN
mycobacterium NVLSFMRYGSDG--SVLACVFNFAGAEHRDYRLGLP-
RAGRWREVLNTDATIYHGSGIGN
Streptomyces NVFAFLRFARDG--
SPLLCVSNFSPVVRHGYRIGVPQEVGQWREVLNTDLEPYGGSGVHH
E. SVLIFVRRDKEG--NEIIVASNFTPVPRHDYRFGIN-
QPGKWREILNTDSMHYHGSNAGN

H.influenzae SVLAFERRSSNG--ERIIVVSNFTPVPRHNYRIGVN-
VAGKYEEILNTDSMYYEGSNVGN
Agro.tume SVFAWLRTAPGE--KPVAVICNLTPVYRENYYVPLG-
VAGRWREILNTDAEIYGGSGKGN
Aquifex_a. SVISFLRKDKSGK-EIILVVCNFTPVPRYDYRVGVP-
KGGYWREIMNTDAKEYWGSGMGN
Synechococcus SIVSFIRRAHESD-RFLVVVCNFTPQPHAHYRIGVP-
VAGFYREIFNSDARSYGGSNMGN
Synechocystis SVVSFIRRAKNSA-EFVVTICNFTPQPHSHYRVGVP-
VPGFYTELFNSDARQYGGSNMGN
Butyrivibrio STYSFYRRASNGK-DNILFVLNMTPMERKGFKVGVP-
FDGTYTKILDSAKECYGGSGSSV
CHLAMYDIA GVVAYLRFADADAKKALLCVHHFGVGYFPHYLLPIL-
PLESCDLLMNTDDTRFGGSGKGF
. :* : :: : . : :::: : **.

Bstearothermophilus GKR-LSAFNEPFHGK----------P--
YHVRMTIPPFGISILRPVQKRGERKRNEK
Bcaldolyticus GKR-LPAFSEPFHGK----------P--
YHVRMTIPPFGISILRPVQKRGERKQNEE
Bsubtilis KKP-LSAKKGALHHK----------P--
CYITMTIPPYGISILRAVKKRGEIKR---
mycobacterium LGG-VDATDDPWHGR----------P--
ASAVLVLPPTSALWLTPA----------
Streptomyces ARA-LRPEPVPAQGR----------A--VSLRMTLPPMATVWLRP----
----
E. GGT-VHSDEIASHGR----------Q--HSLSLTLPPLATIWLVREAE-------
--
H.influenzae FGC-VASEQIESHGR----------E--NSISVSIPPLATVYLRLKTK--
----
Agro.tume GG----RVQAVDAGG----------E--IGAMLVLPPLATIMLEPEN-----
-----
Aquifex_a. LGG-KEADKIPWHGR----------K--FSLSLTLPPLSVIYLKHEG---
----

```
Synechococcus       LGG-KWTDEWSCHNR----------P--
YSLDLCLPPLTTLVLELASGPES----LS
Synechocystis       LGG-KWTEEWSFHEQ----------P--
YSLDLCLPPLSVLVLKLSQNAEENTVPAE
Butyrivibrio        PDK-IKAVKGLCDYK----------D--
YSIEFDLPPYGAEVFVFQTKKTKN-----
CHLAMYDIA
REPEILTPEIARQEREAAGLIEADDESGPDCWGLDIELPPSATLIFSVTLQ---------
                                    . :**   :

Bstearothermophilus   EMHRHVIGRRARKSASLADDKHR----------
Bcaldolyticus         EVHRHVIGRRARKPASLADEKHRETSRAVWGEVPDH
Bsubtilis             ----------
mycobacterium         ----------
Streptomyces          ----------
E.                    ----------
H.influenzae          ----------
Agro.tume             ----------
Aquifex_a.            ----------
Synechococcus         EAANSPL----------
Synechocystis         EASNIA----------
Butyrivibrio          ----------
CHLAMYDIA             ----------
```

Nucleotide sequence of *T. thermophilus* AMase

1 ATGGAGCTTC CCCGCGCTTT CGGTCTGCTT CTCCACCCCA CGAGCCTCCC CGGCCCCTAC
61 GGCGTCGGCG TCCTGGGCCA GGAGGCCCGG GACTTCCTCC GCTTCCTCAA GGAGGCAGGG
121 GGGCGGTACT GGCAGGTCCT CCCCTTGGGC CCCACGGGCT ATGGCGACTC CCCCTACCAG
181 TCCTTCAGCG CCTTCGCCGG AAACCCCTAC CTCATAGACC TGAGGCCCCT CGCGGAAAGG
241 GGCTACGTGC GCCTGGAGGA CCCCGGCTTC CCCCAAGGCC GGGTGGACTA CGGCCTCCTC
301 TACGCCTGGA AGTGGCCCGC CCTGAAGGAG GCCTTCCGGG GCTTCAAGGA AAAGGCCTCC
361 CCGGAGGAGC GGGAGGCCTT CGCCGCCTTC CGGGAGAGGG AGGCCTGGTG GCTCGAGGAC
421 TACGCCCTCT TCATGGCCCT GAAGGGGGCG CACGGGGGGC TTCCCTGGAA CCGGTGGCCC
481 CTTCCCCTGC GGAAGCGGGA AGAGAAGGCC CTTAGGGAGG CGAAAAGCGC CTTGGCCGAG
541 GAGGTGGCCT TCCACGCCTT CACCCAGTGG CTCTTCTTCC GCCAGTGGGG GGCCTTGAAG
601 GCGGAGGCCG AGGCGTTGGG CATCCGGATC ATCGGGGACA TGCCCATCTT CGTGGCCGAG
661 GACTCCGCCG AGGTCTGGGC CCACCCCGAG TGGTTTCACC TGGACGAGGA GGGCCGCCCC
721 ACGGTGGTGG CGGGGGTGCC CCCCGACTAC TTCTCGGAGA CGGGCCAGCG CTGGGGCAAC
781 CCCCTTTACC GCTGGGACGT TTTGGAGCGG GAGGGGTTCT CCTTCTGGAT CCGCCGTCTG
841 GAGAAGGCCC TGGAGCTCTT CCACCTGGTG CGCATAGACC ACTTCCGCGG CTTTGAGGCC
901 TACTGGGAGA TCCCCGCAAG CTGCCCCACG GCGGTGGAGG GGCGCTGGGT CAAGGCCCCG
961 GGGGAGAAGC TCTTCCAGAA GATCCAGGAG GTCTTCGGCG AGGTCCCCGT CCTCGCCGAG
1021 GACCTGGGGG TCATCACCCC CGAGGTGGAG GCCCTGCGCG ACCGCTTCGG CCTTCCCGGG
1081 ATGAAGGTCC TGCAGTTCGC CTTTGACGAC GGGATGGAAA ACCCCTTCCT CCCCCACAAC
1141 TACCCTGCCC ACGGCCGGGT GGTGGTCTAC ACCGGCACCC ACGACAACGA CACCACCCTG
1201 GGCTGGTACC GCACGGCCAC CCCCCACGAG AAGGCCTTCA TGGCGCGGTA CCTGGCGGAC
1261 TGGGGGATCA CCTTCCGGGA AGAGGAGGAG GTGCCCTGGG CCCTGATGCA CCTGGGGATG
1321 AAGTCCGTGG CCCGGCTCGC CGTCTACCCG GTGCAGGACG TCCTGGCCCT GGGCAGCGAG
1381 GCCCGGATGA ACTACCCGGG AAGGCCCTCG GGGAACTGGG CCTGGCGGCT CCTCCCGGGG

1441 GAGCTTTCCC CGGAGCACGG GGCGAGGCTT AGGGCCATGG
CCGAGGCCAC GGAACGGCTC
1501 TAG

Amino acid sequence of *T. thermophilus* AMase

1 MELPRAFGLL LHPTSLPGPY GVGVLGQEAR DFLRFLKEAG GRYWQVLPLG
PTGYGDSPYQ
61 SFSAFAGNPY LIDLRPLAER GYVRLEDPGF PQGRVDYGLL YAWKWPALKE
AFRGFKEKAS
121 PEEREAFAAF REREAWWLED YALFMALKGA HGGLPWNRWP
LPLRKREEKA LREAKSALAE
181 EVAFHAFTQW LFFRQWGALK AEAEALGIRI IGDMPIFVAE DSAEVWAHPE
WFHLDEEGRP
241 TVVAGVPPDY FSETGQRWGN PLYRWDVLER EGFSFWIRRL EKALELFHLV
RIDHFRGFEA
301 YWEIPASCPT AVEGRWVKAP GEKLFQKIQE VFGEVPVLAE DLGVITPEVE
ALRDRFGLPG
361 MKVLQFAFDD GMENPFLPHN YPAHGRVVVY TGTHDNDTTL
GWYRTATPHE KAFMARYLAD
421 WGITFREEEE VPWALMHLGM KSVARLAVYP VQDVLALGSE
ARMNYPGRPS GNWAWRLLPG
481 ELSPEHGARL RAMAEATERL

Nucleotide sequence of *A. aeolicus* MTase

1 ATGAGATTGG CAGGTATTTT ACTTCACGTA ACTTCACTTC CCTCTCCTTA CGGGATAGGG
61 GATCTCGGAA AAGAAGCCTA CAGGTTTCTG GACTTCTTAA AGGAGTGCGG TTTTAGCCTT
121 TGGCAGGTTC TACCTCTGAA CCCCACTTCA CTTGAGGCGG GAAACTCACC CTACAGTTCA
181 AACTCCCTCT TCGCGGGCAA TTACGTACTA ATAGACCCTG AAGAATTATT GGAGGAGGAC
241 TTAATAAAAG AAAGGGACTT AAAAAGATTT CCCTTGGGTG AAGCCCTTTA CGAAGTCGTG
301 TACGAGTATA AAAAAGAGTT GCTCGAAAAA GCCTTTAAAA ATTTCAGGAG ATTTGAACTG
361 CTTGAAGATT TTCTGAAGGA ACACTCTTAC TGGCTCAGAG ATTACGCACT TTACATGGCT
421 ATAAAAGAAG AAGAGGGAAA GGAGTGGTAT GAATGGGATG AAGAATTGAA GAGGAGAGAA
481 AAAGAGGCTT TAAAAAGGGT GTTAAATAAG TTAAAGGGGA GGTTTTACTT CCACGTATTC
541 GTCCAGTTTG TTTTCTTCAA GCAGTGGGAA AAACTGAGAA GATACGCAAG GGAAAGGGGG
601 ATAAGCATAG TTGGAGATCT TCCAATGTAC CCCTCGTACT CAAGTGCGGA CGTGTGGACA
661 AATCCTGAAC TTTTTAAACT GGACGGAGAT TTAAAACCCC TTTTTGTAGC GGGTGTTCCT
721 CCTGATTTTT TCAGTAAAAC GGGACAGCTG TGGGGAAATC CCGTTTACAA CTGGGAAGAA
781 CACGAAAAGG AAGGCTTCAG ATGGTGGATA AGGAGAGTTC ATCACAACTT AAAACTCTTT
841 GACTTTTTAA GACTTGACCA CTTCAGGGGA TTTGAGGCGT ACTGGGAGGT TCCTTACGGT
901 GAAGAAACTG CGGTAAACGG AAGGTGGGTA AAGGCTCCCG GAAAGACACT ATTTAAAAAA
961 CTCTTATCAT ACTTCCCGAA GAACCCATTC ATAGCGGAGG ACTTAGGTTT TATAACGGAC
1021 GAAGTGAGGT ACTTGAGGGA AACTTTTAAA ATCCCGGGAA GCAGAGTTAT TGAGTTTGCC
1081 TTCTACGATA AGGAAAGTGA GCACCTTCCC CACAACGTTG AAGAGAACAA CGTTTACTAC
1141 ACTTCAACTC ATGACCTTCC TCCGATAAGA GGATGGTTTG AGAATTTAGG AGAAGAATCA
1201 AGAAAACGAT TATTTGAATA CTTGGGAAGG GAGATTAAAG AGGAAAAAGT TAACGAGGAG
1261 CTTATAAGAC TCGTTTTAAT CTCAAGGGCG AAGTTCGCAA TAATCCAGAT GCAGGACTTA
1321 CTCAATCTCG GCAATGAAGC GAGGATGAAT TACCCCGGAA GACCTTTCGG AAATTGGAGG
1381 TGGAGAATAA AGGAAGATTA CACACAAAAG AAGGAATTTA TTAAAAAACT CCTCGGAATT
1441 TACGGAAGAG AAGTTTAA

Amino acid sequence of *A. aeolicus* MTase

1 MRLAGILLHV TSLPSPYGIG DLGKEAYRFL DFLKECGFSL WQVLPLNPTS LEAGNSPYSS
61 NSLFAGNYVL IDPEELLEED LIKERDLKRF PLGEALYEVV YEYKKELLEK AFKNFRRFEL
121 LEDFLKEHSY WLRDYALYMA IKEEEGKEWY EWDEELKRRE KEALKRVLNK LKGRFYFHVF
181 VQFVFFKQWE KLRRYARERG ISIVGDLPMY PSYSSADVWT NPELFKLDGD LKPLFVAGVP
241 PDFFSKTGQL WGNPVYNWEE HEKEGFRWWI RRVHHNLKLF DFLRLDHFRG FEAYWEVPYG
301 EETAVNGRWV KAPGKTLFKK LLSYFPKNPF IAEDLGFITD EVRYLRETFK IPGSRVIEFA
361 FYDKESEHLP HNVEENNVYY TSTHDLPPIR GWFENLGEES RKRLFEYLGR EIKEEKVNEE
421 LIRLVLISRA KFAIIQMQDL LNLGNEARMN YPGRPFGNWR WRIKEDYTQK KEFIKKLLGI
481 YGREV

Nucleotide sequence of *A. aeolicus* BE

1 ATGAAGAAGT TCAGTCTCAT CAGTGATTAC GACGTTTACC TCTTTAAGGA GGGAACGCAC
61 ACGAGACTTT ACGATAAACT TGGCTCCCAC GTTATAGAAC TAAACGGGAA AAGGTATACC
121 TTCTTTGCGG TTTGGGCACC CCACGCGGAT TACGTATCAC TTATAGGCGA TTTTAACGAA
181 TGGGATAAAG GTTCTACTCC CATGGTAAAG AGGGAGGACG GCTCCGGAAT ATGGGAGGTT
241 TTACTTGAAG GAGACCTGAC TGGTTCAAAG TACAAGTACT TTATAAAGAA CGGGAATTAC
301 GAAGTTGATA AGTCCGATCC CTTCGCATTT TTCTGTGAGC AACCCCCCGG AAACGCTTCC
361 GTAGTGTGGA AGCTCAATTA CAGGTGGAAC GACTCCGAAT ACATGAAAAA GAGGAAAAGA
421 GTAAACTCAC ACGACTCGCC TATATCCATA TACGAAGTTC ACGTGGGTTC TTGGAGGAGA
481 GTTCCAGAAG AGGGAAACAG ATTTTTGAGC TATAGGGAAC TTGCCGAATA CCTCCCATAC
541 TACGTAAAAG AGATGGGATT TACTCACGTT GAGTTCTTAC CCGTTATGGA ACATCCCTTT
601 TACGGCTCTT GGGGCTACCA GATAACGGGC TACTTCGCTC CGACTTCCAG ATACGGAACT
661 CCTCAGGACT TTATGTACTT AATAGACAAA CTTCATCAAG AAGGGATAGG TGTGATACTA
721 GACTGGGTTC CCTCTCACTT TCCCACCGAT GCCCACGGGC TCGCATACTT TGACGGGACT
781 CACCTTTACG AGTACGAGGA CTGGAGAAAG AGGTGGCATC CCGACTGGAA CAGCTTTGTT
841 TTTGATTACG GAAAACCGGA AGTTCGCTCC TTTCTCCTGA GTTCTGCCCA CTTCTGGCTC
901 GACAAGTACC ACGCAGACGG TCTCAGAGTG GATGCAGTTG CTTCAATGCT TTACCTAGAT
961 TACTCTAGGA AAGAATGGGT TCCAAACATA TACGGAGGGA AAGAAAACCT CGAGGCTATA
1021 GAATTCCTCA GGAAGTTTAA CGAAAGCGTT TACAGAAATT TTCCAGACGT CCAGACAATA
1081 GCGGAGGAAT CAACAGCCTG GCCTATGGTG TCCAGACCTA CATACGTGGG GGGACTGGGA
1141 TTTGGAATGA AGTGGAATAT GGGTTGGATG AACGACACAC TCTTTTACTT TTCAAAGGAT
1201 CCCATCTACA GGAAGTACCA CCATGAAGTC CTCACTTTCA GTATATGGTA CGCTTTTTCC
1261 GAGAACTTCG TCCTTCCACT ATCCCACGAT GAAGTTGTTC ACGGAAAGGG TTCTCTGATA
1321 GGGAAGATGC CAGGAGATTA CTGGCAGAAG TTTGCAAACC TTAGAGCCCT TTTCGGATAC
1381 ATGTGGGCAC ACCCAGGGAA AAAACTCCTC TTTATGGGGG GAGAGTTCGG ACAGTTTAAG

1441 GAATGGGATC ACGAAACGAG TCTCGACTGG CACCTCTTGG
AATACCCTTC TCACAGAGGT
1501 ATTCAGAGAT TAGTTAAGGA CTTAAACGAA GTTTACAGGA
GGGAAAAGGC TTTGCACGAA
1561 ACGGATTTTT CACCTGAGGG CTTTGAGTGG GTAGACTTCC
ACGACTGGGA AAAGAGCGTT
1621 ATATCCTTCT TGAGAAAGGA CAAAAGCGGT AAGGAAATTA TACTCGTAGT
TTGCAACTTC
1681 ACACCCGTTC CGAGATACGA TTACAGGGTA GGTGTACCGA
AAGGCGGATA CTGGAGGGAG
1741 ATAATGAATA CCGATGCAAA GGAGTACTGG GGCTCCGGAA
TGGGAAATCT GGGTGGAAAA
1801 GAGGCTGATA AAATCCCGTG GCACGGAAGA AAATTCTCAC TTTCACTTAC
CCTGCCTCCC
1861 CTTTCCGTGA TCTATTTAAA GCACGAAGGA TGA

Amino acid sequence of *A. aeolicus* BE

1 MKKFSLISDY DVYLFKEGTH TRLYDKLGSH VIELNGKRYT FFAVWAPHAD
YVSLIGDFNE
61 WDKGSTPMVK REDGSGIWEV LLEGDLTGSK YKYFIKNGNY EVDKSDPFAF
FCEQPPGNAS
121 VVWKLNYRWN DSEYMKKRKR VNSHDSPISI YEVHVGSWRR
VPEEGNRFLS YRELAEYLPY
181 YVKEMGFTHV EFLPVMEHPF YGSWGYQITG YFAPTSRYGT PQDFMYLIDK
LHQEGIGVIL
241 DWVPSHFPTD AHGLAYFDGT HLYEYEDWRK RWHPDWNSFV
FDYGKPEVRS FLLSSAHFWL
301 DKYHADGLRV DAVASMLYLD YSRKEWVPNI YGGKENLEAI EFLRKFNESV
YRNFPDVQTI
361 AEESTAWPMV SRPTYVGGLG FGMKWNMGWM NDTLFYFSKD
PIYRKYHHEV LTFSIWYAFS
421 ENFVLPLSHD EVVHGKGSLI GKMPGDYWQK FANLRALFGY
MWAHPGKKLL FMGGEFGQFK
481 EWDHETSLDW HLLEYPSHRG IQRLVKDLNE VYRREKALHE
TDFSPEGFEW VDFHDWEKSV
541 ISFLRKDKSG KEIILVVCNF TPVPRYDYRV GVPKGGYWRE IMNTDAKEYW
GSGMGNLGGK
601 EADKIPWHGR KFSLSLTLPP LSVIYLKHEG

Claims

1. An isolated or recombinant nucleic acid derived from a nucleic acid encoding a polypeptide essentially having alpha-glucanotransferase activity but having essentially no hydrolysing activity, said isolated or recombinant nucleic acid encoding a polypeptide with hydrolytic activity.

2. A nucleic acid according to claim 1 wherein said transferase comprises amylomaltase or branching enzyme.

3. A nucleic acid according to claim 1 or 2 wherein said transferase comprises a thermostable transferase.

4. A nucleic acid according to anyone of claims 1 to 3 wherein said transferase is derived from a thermophilic micro-organism.

5. A nucleic acid according to claim 4 wherein said micro-organism comprises *Thermus thermophilus*, *Thermus aquaticus* or *Aquifex aeolicus*.

.

6. A nucleic acid according to anyone of claims 1 to 5 wherein said transferase is known under EC number 2.4.1.25 or 2.4.1.18

7. A nucleic acid according to anyone of claims 1 to 6 provided with a mutation leading to an alteration or loss of a codon originally encoding a hydrophobic amino acid located in or around a acceptor, a donor or a catalytic site extending from a TIM barrel structure of said transferase.

8. A nucleic acid according to claim 7 wherein said codon originally encoding a hydrophobic amino acid is altered into a codon encoding an amino acid which is substantially less hydrophobic.

9. A nucleic acid according to claim 7 or 8 wherein said hydrophobic amino acid comprises phenylalanine, tryptophan or tyrosine.

10. A nucleic acid according to anyone of claims 7 to 9 wherein said hydrophobic amino acid is located at or around an amino acid position essentially corresponding to amino acid position 54, 251, 258 or 366 of amylomaltase of *Thermus thermophilus* HB8.

11. A vector comprising a nucleic acid according to anyone of claims 1 to 10.

12. A host cell comprising a vector according to claim 11 or a nucleic acid according to anyone of claims 1 to 10.

13. A method for providing a polypeptide or fragment thereof essentially having alpha glucanotransferase acitivity but having essentially no hydrolysing activity with hydrolysing activity said method comprising providing a nucleic acid encoding such a transferase with a mutation leading to an alteration or loss of a codon originally encoding a hydrophobic amino acid located in or around a acceptor, a donor or a catalytic site extending from a (alphalpha/betaeta)$_8$ barrel structure of said transferase.

14. A polypeptide, or an enzymatically functional fragment thereof encoded by a nucleic acid according to anyone of claims 1 to 10 or obtainable by a method according to claim 13.

15. Use a polypeptide or fragment according to claim 14 in reducing retrogradation of starch.

16. Use according to claim 15 in reducing retrogradation of amylopectine.

17. Use according to claim 16 in reducing long-term retrogradation of amylpectine.

18. Use a polypeptide or fragment according to claim 14 in hydrolysing starch.

19. A method for reducing retrogradation of starch comprising treating said starch

with a polypeptide or fragment according to claim 14.

20. A method for hydrolysing starch comprising treating said starch with a polypeptide or fragment according to claim 14.

21. A bakery ingredient comprising a polypeptide or fragment according to claim 14.

22. A bakery product such as bread comprising a polypeptide or fragment according to claim 14.

FIGURE 1

FIGURE 2

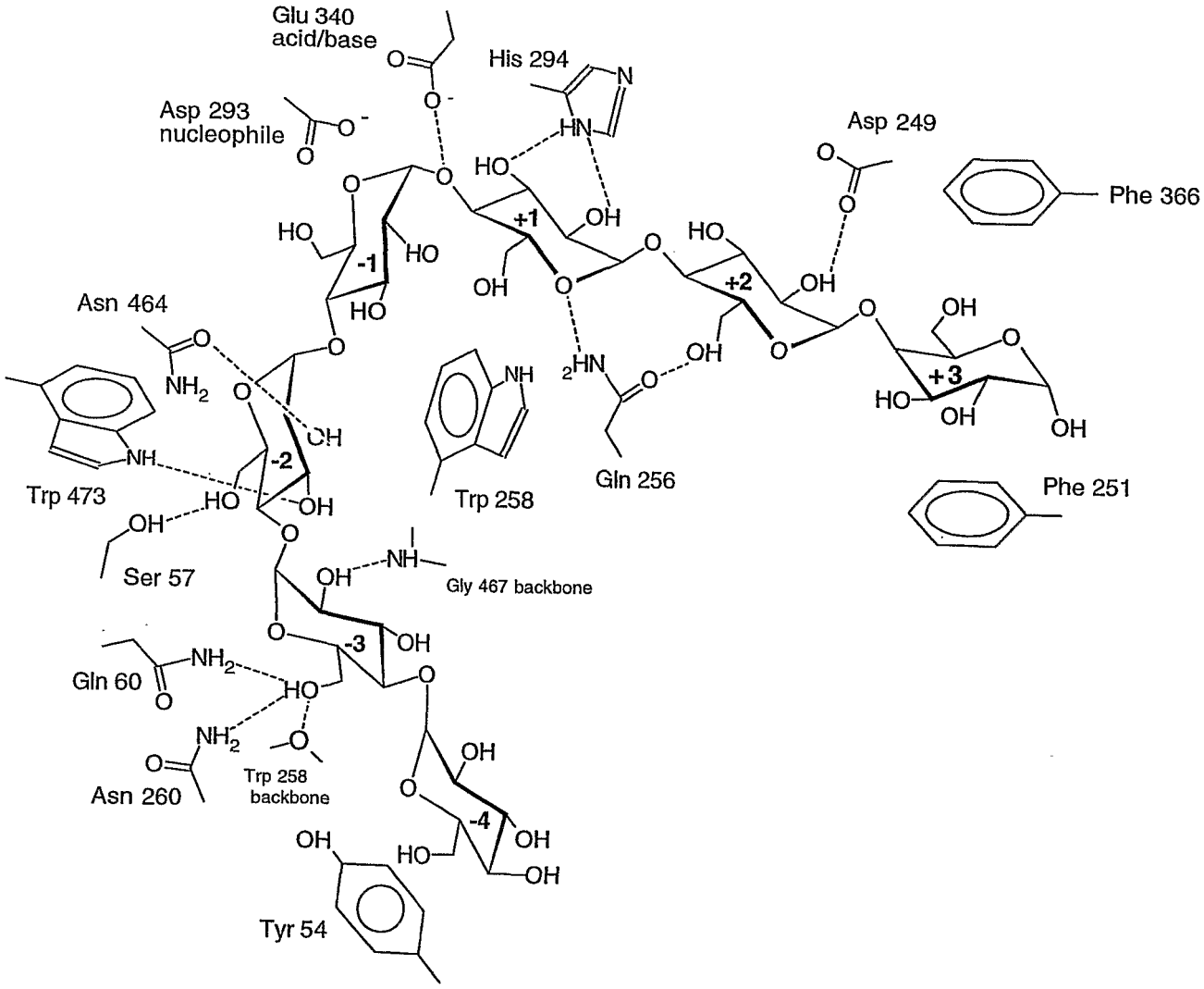

Glu 340
acid/base
His 294
Asp 293
nucleophile
Asp 249
Phe 366
Asn 464
Trp 473
Trp 258
Gln 256
Phe 251
Ser 57
Gly 467 backbone
Gln 60
Asn 260
Trp 258
backbone
Tyr 54
-1
+1
+2
+3
-2
-3
-4

FIGURE 3

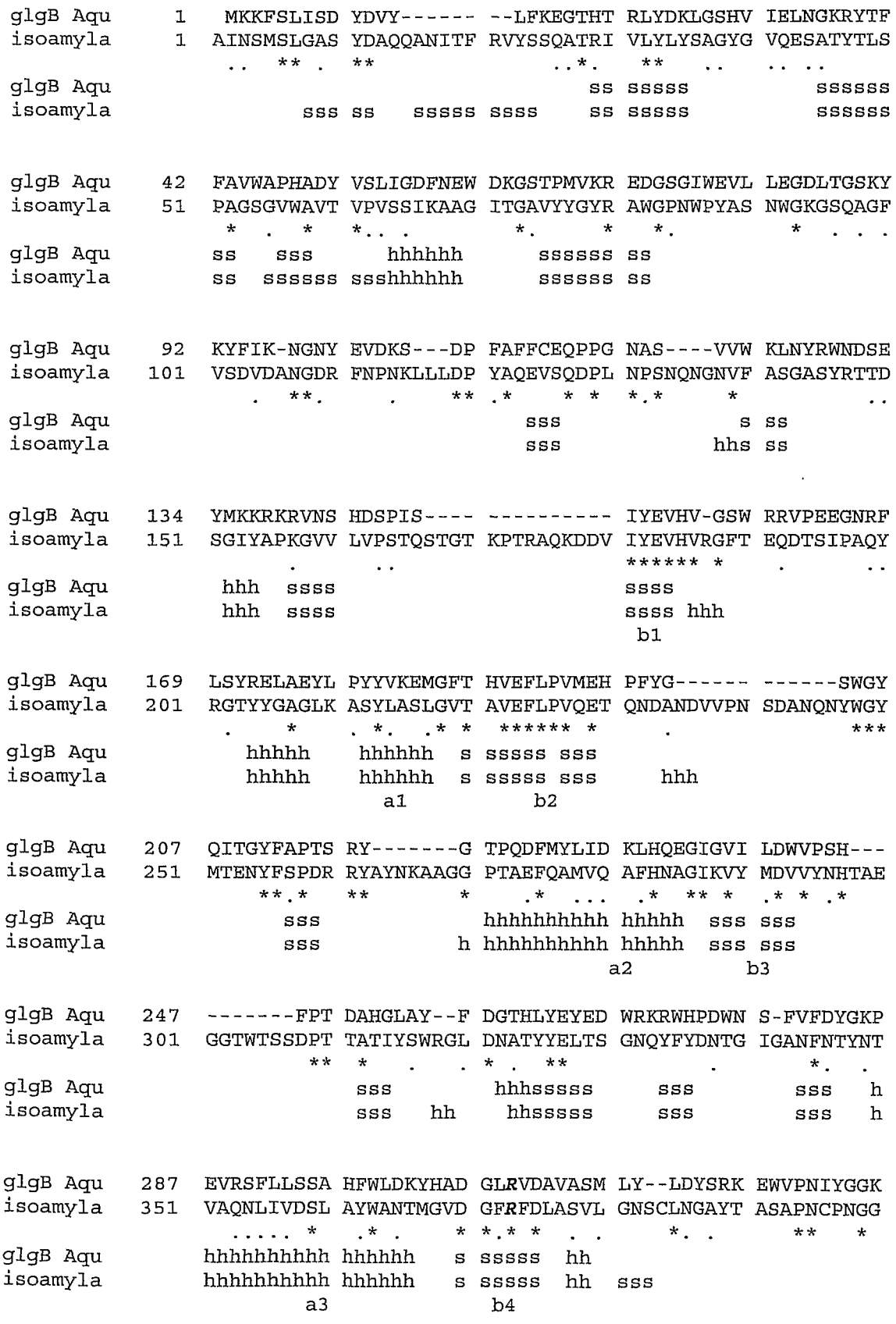
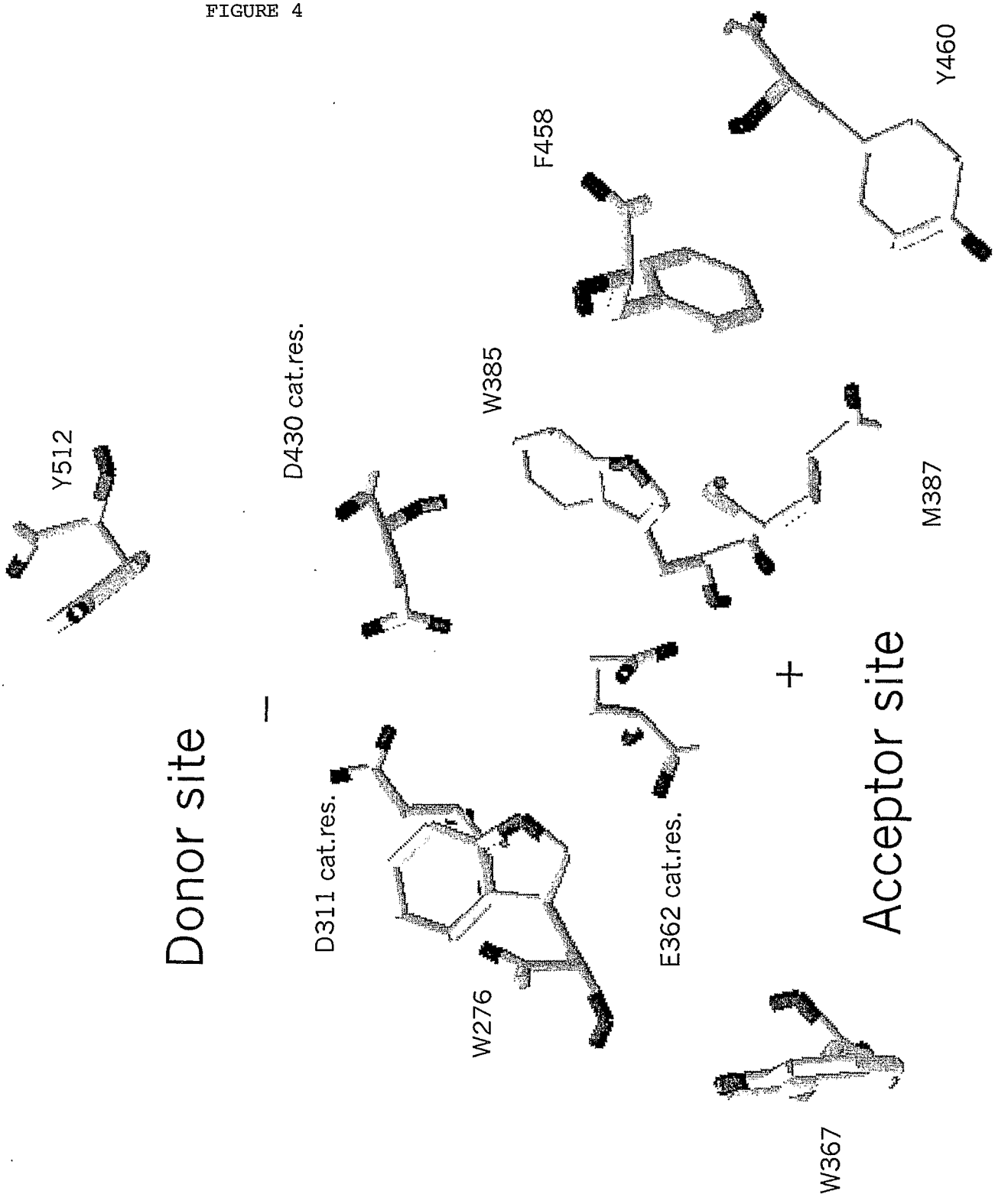

glgB Aqu 1 MKKFSLISD YDVY------ --LFKEGTHT RLYDKLGSHV IELNGKRYTF
isoamyla 1 AINSMSLGAS YDAQQANITF RVYSSQATRI VLYLYSAGYG VQESATYTLS
glgB Aqu ss sssss ssssss
isoamyla sss ss sssss ssss ss sssss ssssss
glgB Aqu 42 FAVWAPHADY VSLIGDFNEW DKGSTPMVKR EDGSGIWEVL LEGDLTGSKY
isoamyla 51 PAGSGVWAVT VPVSSIKAAG ITGAVYYGYR AWGPNWPYAS NWGKGSQAGF
glgB Aqu ss sss hhhhhh ssssss ss
isoamyla ss ssssss ssshhhhhh ssssss ss
glgB Aqu 92 KYFIK-NGNY EVDKS---DP FAFFCEQPPG NAS----VVW KLNYRWNDSE
isoamyla 101 VSDVDANGDR FNPNKLLLDP YAQEVSQDPL NPSNQNGNVF ASGASYRTTD
glgB Aqu sss s ss
isoamyla sss hhs ss
glgB Aqu 134 YMKKRKRVNS HDSPIS---- ---------- IYEVHV-GSW RRVPEEGNRF
isoamyla 151 SGIYAPKGVV LVPSTQSTGT KPTRAQKDDV IYEVHVRGFT EQDTSIPAQY
glgB Aqu hhh ssss ssss
isoamyla hhh ssss ssss hhh
b1
glgB Aqu 169 LSYRELAEYL PYYVKEMGFT HVEFLPVMEH PFYG------ ------SWGY
isoamyla 201 RGTYYGAGLK ASYLASLGVT AVEFLPVQET QNDANDVVPN SDANQNYWGY
glgB Aqu hhhhh hhhhhh s sssss sss
isoamyla hhhhh hhhhhh s sssss sss hhh
a1 b2
glgB Aqu 207 QITGYFAPTS RY-------G TPQDFMYLID KLHQEGIGVI LDWVPSH---
isoamyla 251 MTENYFSPDR RYAYNKAAGG PTAEFQAMVQ AFHNAGIKVY MDVVYNHTAE
glgB Aqu sss hhhhhhhhhh hhhhh sss sss
isoamyla sss h hhhhhhhhhh hhhhh sss sss
a2 b3
glgB Aqu 247 -------FPT DAHGLAY--F DGTHLYEYED WRKRWHPDWN S-FVFDYGKP
isoamyla 301 GGTWTSSDPT TATIYSWRGL DNATYYELTS GNQYFYDNTG IGANFNTYNT
glgB Aqu sss hhhssssss sss sss h
isoamyla sss hh hhssssss sss sss h
glgB Aqu 287 EVRSFLLSSA HFWLDKYHAD GLRVDAVASM LY--LDYSRK EWVPNIYGGK
isoamyla 351 VAQNLIVDSL AYWANTMGVD GFRFDLASVL GNSCLNGAYT ASAPNCPNGG
glgB Aqu hhhhhhhhhh hhhhhh s sssss hh
isoamyla hhhhhhhhhh hhhhhh s sssss hh sss
a3 b4

FIGURE 3, Contd.

```
glgB Aqu    335  ENLEAIEFLR KFN----ESV YR---NFPDV QTIAEESTAW PMVSRPTYVG
isoamyla    401  YNFDAADSNV AINRILREFT VRPAAGGSGL DLFAEPWAIG G--NSYQLGG
                  *..* .     .*    *    *     . . . .** .      .     *
glgB Aqu                                          sssss
isoamyla           sss       hhhhh                sssss
                               a4                    b5

glgB Aqu    378  GLGFGMKWNM GWMNDTL--- --FYFSKDPI YRKYHHEVLT FSIWYAFS--
isoamyla    449  FPQGWSEWNG LFR-DSLRQA QNELGSMTIY VTQDANDFSG SSNLFQSSGR
                       **      *.*         *.          ..     *  .  *
glgB Aqu              ssss  hh                    hhhhhhhh    hhhhhh
isoamyla              ssss  hhh hhhhhh hh          hhhhhhhh    hhhhhh
                       b6                                      a6

glgB Aqu    421  ---ENFVLPL SHD-EVVHGK GSLIGKMPGD YWQKFANLR- ALFGYMWAHP
isoamyla    498  SPWNSINFID VHDGMTLKDV YSCNGANNSQ AWPYGPSDGG TSTNYSWDQG
                    .... .    **   .     *  *   ..  *   ..    .   * * .
glgB Aqu              ssss         hhh                                s
isoamyla              ssss         hhh                                s
                       b7

glgB Aqu    466  GKKLLFMGGE FGQFKEWDHE TSLDWHLLEY PSHRG---IQ RLVKDLNEVY
isoamyla    548  MS--AGTGAA VDQRRAARTG MAFEMLSAGT PLMQGGDEYL RTLQCNNNAY
                        *.    * .       ...       *  .*      * .   *. *
glgB Aqu         ss  ssss h hhhhhhhhhh hhhhhh   s sssss
isoamyla         ss   sss h hhhhhhhhhh hhhhhh   s sssss
                                    a7           b8

glgB Aqu    513  RREKALHETD FSPEGFEWVD FHDWEKSVIS FLRKDKSGKE IILVVCNFTP
isoamyla    596  NLDSSANWLT YS-WTTDQSN FYTFAQRLIA FRKAHPALRP SSWYSG--SQ
                   . . .    .*    . . *      .*. * .   . .          .
glgB Aqu                        hhhhh hhhhhhhhhh hhhh               s
isoamyla                        hhhhh hhhhhhhhhh hhhh               s
                                      a8

glgB Aqu    563  VPRYDYRVGV PKGGYWREIM N-TDAKEYWG SGMGNLGGKE ADKIPWHGRK
isoamyla    643  LTWYQPSGAV ADSNYWNNTS NYAIAYAING PSLGDSNSIY VAYNGWSSSV
                 . .*.   .* . . ** .   * . *   * ...*.  .        * .
glgB Aqu         sssss   ss s hhhhh      ssssss  hhh     ss sss
isoamyla         sssss   ss s hhhhh     ssssssss hhh    sss sssss    s

glgB Aqu    612  FSLSLTLPPL SVIYLKHEG
isoamyla    693  TFTLPAPPSG TQWYRVTDTC DWNDGASTFV APGSETLIGG AGTTYGQCGQ
                       . *.  .  *   .
glgB Aqu
isoamyla         ssss        ssssssss                  sss     sssss

glgB Aqu
isoamyla    743  SLLLLISK

glgB Aqu
isoamyla         ssssssss
```

FIGURE 4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D430 cat.res.
D311 cat.res.
W276
W385
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Y460
E362 cat.res.
M387
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